MECHANISM OF BIOMATERIAL ADJUVANT EFFECT: PHENOTYPE OF DENDRITIC CELLS UPON BIOMATERIAL CONTACT

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MECHANISM OF BIOMATERIAL ADJUVANT EFFECT:
PHENOTYPE OF DENDRITIC CELLS UPON BIOMATERIAL CONTACT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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SUMMARY

Development of tissue engineering to address the clinical shortage of donor organs for transplantation has advanced the study of biomaterials. Such biomaterials are currently being explored for a variety of applications including combination products in which both biological and biomaterial components together are introduced to the host upon implantation. Significant progress has been made in the development of new biomaterials and modifications to improve biocompatibility, and in the understanding of the host inflammatory response elicited upon introduction of a biomaterial. However, investigation of the potential of biomaterials to increase the specific immune response against antigens associated with the biological component, through adjuvant effect, is yet to be fully explored.

The objective of this thesis research was to evaluate the adjuvant effect of biomaterials by focusing on their effects on dendritic cell (DC) phenotype upon contact. As DC maturation is manifest of adjuvant effect and leads to initiation of adaptive immune response, the predisposition of biomaterials to induce DC maturation *in vitro* would be predicative of how biomaterials would perform *in vivo* upon implantation. Specifically, the differential effects of two model biomaterials used in combination products, agarose and 75:25 poly(lactic-*co*-glycolic acid) (PLGA) were evaluated. These biomaterials were chosen for comparison since adjuvant effect has been observed with PLGA (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001; Matzelle and Babensee, 2004), whereas agarose has been shown to induce minimal inflammatory response (Starke et al., 1987; Rahfoth et al., 1998). It was hypothesized that these biomaterials would exhibit differential effects on DC maturation, and this biomaterial-

induced maturation would be mediated by receptors and mechanisms similar to those used by DCs in response to pathogen-induced activation of these cells.

Following initial characterization of agarose and PLGA in microparticle (MP) and film forms to be used, cell culture system was established for deriving DCs from human peripheral blood mononuclear cells. Treatment of immature DCs (iDCs) with MPs or films of PLGA resulted in maturation of DCs as measured by increased expression of co-stimulatory and MHC molecules, allostimulatory capacity, and induction of delayed type hypersensitivity. The extent of PLGA-induced maturation phenotype was in between that of iDCs and DCs matured with a known DC maturation stimulant, lipopolysaccharide (LPS). Moreover, PLGA MP-induced DC maturation was demonstrated to require direct contact between DCs and PLGA MPs, and not mediated by autocrine effects of pro-inflammatory cytokine, tumor necrosis factor α (TNFα).

As phagocytosis by DCs may contribute to their maturation, molecular aspects of MP phagocytosis by DCs were evaluated. Treating iDCs with PLGA or polystyrene fluorospheres of approximately 3 μm in diameter resulted in the internalization of the particles as evidenced by confocal laser scanning micrographs. This uptake of fluorospheres by DCs was decreased by pretreatment of cells with cytochalasin D or by incubation with the fluorospheres at 4°C, and was sensitive to EDTA and trypsin pretreatments in a dose-dependent manner. In agreement with previous studies, treatment of iDCs with PLGA MPs but not with polystyrene MPs led to increased release of TNFα, which was dependent on the ratio of PLGA MPs to DCs.

The differential effects of agarose and PLGA were established by directly comparing DC maturation in response to agarose or PLGA films. These experiments

revealed that DCs treated with PLGA film expressed higher levels of co-stimulatory and MHC class II molecules, were more allostimulatory, and secreted higher amounts of pro-inflammatory cytokines. These results imply that PLGA but not agarose is a stimulus of DC maturation. In contrast, activation of nuclear transcription factor, nuclear factor κ B, (NFκB) measured 24 hrs after the initial stimulation of DCs with agarose or PLGA films revealed that while both agarose- and PLGA-treated DCs increased activation of NFκB above that of iDCs, agarose-treated DCs showed higher activation of all members of NFκB transcription factor family. While this difference was not explored in the study presented herein, it is hypothesized that PLGA-induced activation of NFκB peaks at earlier time point, and is waned by 24 hrs, whereas agarose induces NFκB activation at a later time point, still measurable at 24 hrs post-treatment.

To further investigate the mechanisms of PLGA-induced DC maturation, roles of various receptors that are involved in signaling for DC maturation and uptake of MPs were assessed. In particular, efforts were focused on the elucidation of the role of Toll-like receptors 2 (TLR2) and 4 (TLR4) as ligation of these receptors by endogenous and exogenous adjuvants has been shown to lead to DC activation. While the expression of CD14, CD36, CD51, DC-SIGN, mannose receptor, TLR2, and TLR4 on human iDCs was confirmed by flow cytometry, blocking of these receptors using antibodies or known ligands to these receptors within the range tested was not able to block LPS- or PLGA MP-induced maturation. In addition, blocking TLR2, TLR4, or CD14 either alone or in combination was unable to reduce LPS- or PLGA-induced DC maturation. Pretreatment of DCs with TLR2 antibody alone resulted in enhanced expression of CD83 and CD86 as well as secretion of TNFα without any additional maturation stimulus, suggesting that

deciphering the role of these receptors by *in vitro* blocking experiments was difficult using these particular antibodies.

To circumvent this technical difficulty, PLGA-induced maturation of DCs derived from bone marrows of commercially available murine model with a mutation in toll-like receptor 4 gene, $Tlr4^{Lps\text{-}d}$, C3H/HeJ strain, was assessed. Prior to evaluating the role of TLR4 on PLGA-induced DC maturation, maturation of murine DCs in response to PLGA was confirmed using bone marrow-derived DCs from wild type C57BL6 mice. Bone marrow-derived DCs of C57BL6 mice increased expression of CD86 in response to treatment with LPS, PLGA MP, or PLGA film. In addition, DCs treated with PLGA MPs or films secreted significantly higher amounts of pro-inflammatory cytokines, TNFα and IL-6, than did iDCs. Upon confirming that murine DCs respond to PLGA as do human DCs, experiments were repeated using DCs from C3H/HeJ mice, and compared to those using DCs from its control with the same haplotype, C3H/HeOuJ mice. As expected, DCs from C3H/HeJ mice treated with LPS did not show any difference in expression of maturation markers as compared to iDCs, whereas DCs from C3H/HeOuJ mice showed increased CD86 expression with LPS treatment. Increase in CD86 expression was not induced in response to PLGA MPs or PLGA film for DCs from either of the strains. Similarly, DCs from C3H/HeOuJ mice secreted higher amounts of TNFα and IL-6 as compared to DCs from C3H/HeJ mice in response to LPS. However, DCs from neither strain responded to treatment with PLGA MPs or film, in accord with results from marker expression. The lack of response in C3H/HeOuJ animals as compared to C57BL6 animals is perhaps attributed to the particular haplotypes of these animals, as MHC molecules regulate cytokine production.

Collectively, these results establish the differential adjuvant effects of agarose and PLGA on the level of DC maturation, and begin to elucidate the mechanisms of biomaterial adjuvant effect. In addition, assays developed herein provide methods to screen for biomaterials to be used in combination products, such that biomaterials with desired levels of adjuvanticity as measured by DC maturation effects may be selected for given application.

CHAPTER 1

INTRODUCTION

Despite advancements in transplantation biology, shortage of donor organs remains a limiting factor in the treatment of tissue loss or end-stage organ failure. To address this clinical demand, tissue engineered constructs, comprised of cells of autologous, allogeneic, or xenogeneic in origin, seeded onto a biomaterial scaffold, have emerged as potential alternative with hopes of achieving biological integration as a functional organ (Langer and Vacanti, 1993; Atala, 2004; Kulig and Vacanti, 2004; Tseng et al., 2005). While such engineered constructs, some of which have already been approved by federal agencies but most of which are still in the research and developmental stages, have shown promise, success has been in part limited by the host immune and inflammatory responses towards the device (Babensee et al., 1998; Llull, 1999; Lavik and Langer, 2004). Unlike the response against transplanted organs, the presence of the biomaterial component in a tissue engineered device and its associated inflammatory responses may have an adjuvant effect and potentiate the specific immune response against the cellular component of the device, thereby compromising device effectiveness (Babensee et al., 1998). The use of biomaterials in diverse applications in which the intended functions for the biomaterial and its adjuvant effect differ substantiates the need to understand the host responses associated with the biomaterial for appropriate material design and selection depending on the application. For example, biomaterials with minimal adjuvant effect are desirable for tissue engineering

applications, whereas those with high adjuvant effect are desirable for non-viral vaccine delivery systems to improve antigen immunogenicity.

The objective of this thesis research was to evaluate the adjuvant effect of biomaterials by focusing on their effects on dendritic cell phenotype upon contact, as maturation of dendritic cells is manifest of adjuvant effect, and leads to activation of the adaptive immune response. In particular, the effect of biomaterial contact on dendritic cell maturation and receptors and mechanisms involved in this biomaterial-induced maturation were investigated. **It was hypothesized that biomaterial contact with immature dendritic cells induces their maturation via receptors and mechanisms similar to those used in response to pathogen-induced activation of these cells, enhancing their T cell stimulatory capacity.** While host response against biomaterials and the application of biomaterials as vaccine adjuvants have been studied extensively, the adjuvant effect of biomaterials on dendritic cell maturation in the context of combination products remains largely unexplored. Biomaterials such poly(lactic-*co*-glycolic acid) (PLGA) are capable of producing an adjuvant effect when used as a carrier vehicle for model antigens, as measured by production of cytokines by T cells (Newman et al., 1998; Stivaktakis et al., 2005), as well as by increase in the production of antigen-specific antibody (Matzelle and Babensee, 2004) depending on the form of the biomaterial (Bennewitz and Babensee, 2005) and processes of biomaterial carrier formulation (Jaganathan et al., 2004). Because adjuvants act on dendritic cells and induce their co-stimulatory activity to effect increased antigen immunogenicity, the understanding of the effect of biomaterial adjuvants on dendritic cell maturation is central to the appropriate design of biomaterials for use in wide range of applications.

To address the above central hypothesis, two specific aims were pursued.

Specific aim 1: Demonstrate that biomaterial contact induces dendritic cell maturation *in vitro* and *in vivo*.

The working hypothesis for this aim was that biomaterial contact promotes maturation of dendritic cells *in vitro* and *in vivo* to become efficient antigen presenting cells as characterized by increased expression of co-stimulatory and MHC molecules, cytokine secretion, and allogeneic T cell stimulatory capacity. For this aim, immature dendritic cells were generated *in vitro*, and treated with biomaterials commonly used in tissue engineering, namely 75:25 poly(lactic-co-glycolic acid) or agarose. These particular biomaterials were chosen for comparison as PLGA has been shown to have adjuvant effect (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001; Matzelle and Babensee, 2004), whereas agarose has been shown to induce minimal inflammatory response (Starke et al., 1987; Rahfoth et al., 1998). Through comparison of these two biomaterials, which seemingly have dissimilar adjuvant effect, we aimed to substantiate the differential biomaterial adjuvanticity, by focusing on the maturation of dendritic cells. Dendritic cell maturation was assessed by cell morphology, surface marker expression, cytokine release, and allostimulatory activity in a mixed lymphocyte reaction. Further, biomaterial-induced dendritic cell maturation *in vivo* was measured as delayed type hypersensitivity reaction to a model antigen delivered with biomaterial microparticles.

Specific aim 2: Identify mechanisms/ receptors involved in the biomaterial-induced dendritic cell maturation.

Several aspects of dendritic cell maturation mechanisms upon biomaterial contact were investigated. Contribution of direct biomaterial contact and phagocytosis, role of known pattern recognition receptors for biomaterial-induced dendritic cell maturation, and subsequent cellular functions including the activation of nuclear transcription factor NFκB and release of inflammatory cytokines were evaluated. It was hypothesized that dendritic cell maturation and its mechanisms are dependent on the form of the biomaterial in contact with the cells and is in part mediated by the recognition of the biomaterial surface or adsorbed proteins by pattern recognition receptors on dendritic cells.

Collectively, these studies begin to elucidate the mechanisms of differential biomaterial adjuvant effect. Results presented herein provide unique characterization approaches for evaluation of host response to biomaterials by focusing on the adjuvant effect, in particular, at the level of dendritic cell maturation, an essential step in orchestrating subsequent adaptive immune responses. Especially with the advances in combination product development, understanding the interaction between innate inflammation against biomaterials and adaptive immunity against biological components is central to the control of biomaterial design to achieve desirable host immune responses.

CHAPTER 2

RESEARCH SIGNIFICANCE

In 2002, in the United States alone, almost 81,000 people were on the waiting list for an organ transplant, while the actual number of total transplants was less than 23,000 (Transplant Statistics: National Reports, Scientific Registry of Transplant Recipients). To address current limitations of organ transplantation such as sourcing and compatibility, tissue engineering has evolved as a potential alternative to organ transplantation (Langer and Vacanti, 1993; Stock and Vacanti, 2001); however, the recipient immune response elicited against a tissue engineered device remain a hindrance to the successful integration of engineered grafts (Babensee et al., 1998; Llull, 1999; Lavik and Langer, 2004). Such response likely includes both specific adaptive immune response against the cellular and biological components of a device as well as nonspecific inflammatory response against the carrier matrix, often composed of biomaterials (Babensee et al., 1998). In particular, biomaterials have been widely explored for drug delivery devices and tissue engineered constructs in which the biomaterial component serves various functional and structural purposes (Peppas and Langer, 1994). Current research efforts in biomaterial sciences center on the development of new biomaterials and modifications of biomaterials, to improve biocompatibility, target specificity, and other physicochemical properties (Langer and Tirrell, 2004). However, for combination products in which drug, device, or biologics are present in combination, the added existence of biological components requires that biomaterial properties be appropriately studied in the context of both the biomaterial and biological components.

The host response to combination products is one such important property that is greatly influenced by the simultaneous introduction of biomaterial and biological components. The interplay between immune and inflammatory responses elicited against the biological and the biomaterial components, respectively, can lead to augmented reaction against the entire product, resulting in unexpected device failure (Babensee et al., 1998). In particular, the potential of biomaterials to enhance the specific immune response against antigens from the biological component, through their adjuvant effect, may determine selection and design of an appropriate material for use in combination products. The research presented herein addresses the adjuvant effect of biomaterials by focusing on biomaterial effects on maturation of dendritic cells (DCs), professional antigen presenting cells that bridge innate and adaptive immunity.

While study of biomaterials in the context of tissue engineering and vaccine adjuvants has revolved around inflammatory capacity of materials and production of immunogen-specific antibodies, respectively, the effect of biomaterials on DCs is yet to be fully characterized. The objective of this thesis research was to evaluate the adjuvant effect of biomaterials commonly used in combination products, in particular, their effects on DC maturation, and receptors and mechanisms involved in this biomaterial-induced maturation. Since the inception of this research project, a few investigational studies addressing the role of biomaterials in DC maturation have been reported (Kempf et al., 2003; Sun et al., 2003; Jilek et al., 2004; Waeckerle-Men et al., 2004). However, while these studies have focused on applications of biomaterial/ DC interaction for vaccine delivery to enhance protective immunity, this research project is unique in that we seek to apply the knowledge of biomaterial effect on DC phenotype as screening protocols for

biomaterial development for combination products where varying degrees of adjuvant effect are required. This application includes tissue engineering, where enhanced immune response against the construct is undesirable.

To this end, differential effects of two model biomaterials used in combination products, agarose and poly(lactic-*co*-glycolic acid) (PLGA), on DC maturation was demonstrated; treatment of DCs with synthetic polymer PLGA resulted in DC phenotypes consistent with maturation, whereas treatment with agarose did not. This finding is significant in that it establishes differential effects of biomaterials, substantiating the maturation effect observed with PLGA. Understanding of biomaterial adjuvant effect on DC maturation and its mechanisms, will aid in the definition of design and selection criteria for tissue engineering biomaterials that promote host integration. Moreover, this knowledge can be translated into the development of biomaterial-centered immunomodulatory strategies to control host acceptance of tissue engineered grafts to address some of the current clinical limitations encountered in regenerative medicine.

CHAPTER 3

LITERATURE REVIEW

Innate and adaptive immune responses

Innate immunity

Innate immunity is the first line of defense upon invasion of a host by a pathogen that compromises the epithelium, and is mediated by cells and components of the immune system already existing as a part of the host defense mechanism. These pathogens are encountered by tissue-resident phagocytic cells such as the macrophages which recognize molecular patterns associated with the pathogens through their surface receptors (Medzhitov and Janeway, 1997). This recognition initiates the innate immune response through attempts to clear the pathogen via phagocytosis and release of cytokines and chemokines. These released factors not only directly affect the infected cells, but also have local and systemic effects. For example, some of the cytokines secreted by macrophages and their function include IL-1 which activates the lymphocytes, tumor necrosis factor α (TNFα) which activates the vascular endothelium for increased permeability to allow cell recruitment to the site of infection, and IL-12, which activates natural killer cells to release cytotoxic contents of their granules onto infected cells (Janeway et al., 2001). These cytokines, particularly IL-8, also help attract monocytes and neutrophils from the circulation to the infected tissue to initiate the inflammatory response.

The recognition of pathogens by the cells of innate immunity is accomplished by a set of germline encoded receptors known as pattern recognition receptors (PRRs)

(Medzhitov and Janeway, 1997). These receptors are expressed on epithelial and antigen presenting cells (APCs) and recognize conserved molecular structures on pathogens, termed pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). The recognition of PAMPs by APCs, in particular by immature dendritic cells (iDCs), leads to the activation of these professional APCs, which in turn activates naïve T lymphocytes, resulting in generation of antigen-specific adaptive immunity. Thus DCs have been regarded as the most potent APCs and as a bridge between innate and adaptive immunity (Banchereau and Steinman, 1998).

Another constituent of the innate response includes the complement system. Complements are a set of heat-labile plasma proteins that bind to pathogen surfaces, and undergo enzymatic cleavage to yield soluble anaphylatoxins and surface-bound fragments which mediate inflammatory response and recognition by phagocytes, respectively (Janeway et al., 2001). The three pathways of complement activation are determined by the surface present; the classical pathway is initiated by binding of C1q directly via pathogen surface or indirectly via antibody: antigen binding to the surface, the mannan-binding lectin pathway is initiated by the binding of mannan-binding lectin to bacterial or viral surfaces via mannose-containing carbohydrates, and the alternative pathway is initiated by spontaneous hydrolysis of C3, and subsequent generation and binding of C3b to pathogen surface (Janeway et al., 2001). All three pathways converge at the level of C3 convertase generation, and result in the cleavage of C3 convertase for the generation of anaphylatoxins and peptide mediators of inflammation, C3a, C4a, and C5a. The other fragment of C3 lysis, C3b, is deposited on pathogen surface for opsonization, and is further amplified by the alternative pathway. Successive stages of

the complement cascade common to all pathways lead to the formation of membrane attack complex, composed of C5b, C6, C7, C8, and C9, which creates a pore in the pathogen membrane, resulting in its lysis.

Adaptive Immunity

Activation of APCs such as DCs upon detection of pathogens enables APCs to present the antigen to naïve T lymphocyte, in turn activating them for initiation of the adaptive immune response (Janeway et al., 2001). While adaptive immune response is slower in generation, it provides specificity and memory, via clonal expansion of antigen-specific T lymphocytes. Whereas innate immunity recognizes invading pathogens via PRRs for conserved structures on pathogens, cells of adaptive immunity can recognize a wide repertoire of antigens owing to its ability for recombination of somatic receptor genes. On recognition of a particular antigen presented by APCs in the lymph node, naïve lymphocytes become lymphoblasts and clonally expand. These cells then differentiate into effector cells; B cells secrete antibodies, a secreted form of its receptor (B cell receptor), and T cells function to kill infected cells (CD8+ cytotoxic T cells) or activate other cells of the immune system (CD4+ helper T cells). This activation of lymphocytes however, not only requires the initial recognition of antigens presented by APCs, but also a co-stimulatory signal which can only be provided by professional APCs such as macrophages and DCs. Similarly for B cells, stimulation occurs through the recognition of antigen via B cell receptor, but for activation, signal from activated T cell is also required. While most effector cells undergo apoptosis after a particular antigen has been eradicated, immunological memory is provided by the few memory cells that

persist, which facilitates a quick response in an event there is another encounter with the same antigen (Janeway et al., 2001).

In comparison to humoral immunity implemented by antibodies which can only reach pathogens in blood and extracellular fluid, cell-mediated immune response can directly affect infected host cells (Janeway et al., 2001). Cytotoxic T lymphocytes expressing CD8 on cell surface can recognize infected host cells expressing viral antigens on their surface. Other T lymphocytes expressing CD4 on their surface are helper T cells, and depending on subset, Th1 or Th2, can either activate macrophages to effect antibacterial mechanisms or activate B cells for antibody production. The recognition of antigens by T cells and their activation require that the antigens be processed into peptides that are presented in the context of major histocompatibility complex (MHC) molecules by APCs. The 2 classes of MHC molecules, MHC class I and II, present peptides of a particular origin, and are recognized by CD8+ and CD4+ T cells, respectively. In general, antigens derived in the cytosol, typically of viral origin, are presented in the context of MHC class I, whereas those derived in intracellular vesicles, typically of bacterial origin, are presented in the context of MHC class II molecules. The control of immunity is provided by the ability of only professional APCs to produce co-stimulatory signal (Banchereau and Steinman, 1998). The major co-stimulatory molecules expressed on APCs are CD40 and B7 molecules, CD80 (B7.1) and CD86 (B7.2). Expression of B7 is stimulated by the interaction between CD40 on APCs with CD40 ligand (CD40L) on T cells, further driving T cell stimulation and proliferation (O'Sullivan and Thomas, 2003). For the delivery of co-stimulatory signal, B7 molecules interact with CD28 on T cells, to stimulate clonal expansion of naïve T cells (Janeway et

al., 2001). This proliferation is tightly controlled by a negative regulator, CTLA-4, a CD28-related protein, whose expression increases upon T cell activation. Binding of B7 to CTLA-4 is with higher avidity than the avidity of B7 to CD28, and this ligation sends inhibitory signal to activated T cells. This inhibitory signal controls proliferation by regulating the production of IL-2, autocrine T cell growth factor, and desensitizing naïve T cells to further stimulation by APCs. For the proper induction of adaptive immunity, T cells can become activated only when they receive signal 1 (presentation of antigenic peptide in the context of MHC molecule) and signal 2 (co-stimulation from an APC). T cells receiving signal 1 without signal 2 undergo anergy, being unable to further respond to any signal. Similarly, tolerance to antigens by suppression of antigen-specific response upon rechallenge with the particular antigen is regulated by Th3 and T regulatory cells, both subsets which produce profile of cytokines such as IL-4, IL-10 and transforming growth factor-β (TGF-β) that inhibit the development of Th1 responses and is associated with suppressed inflammatory T cell responses.

Dendritic cells

Immature dendritic cells and antigen capture

Dendritic cells are professional APCs, and differ from other APCs in that they can initiate primary immune responses, and give rise to immunological memory (Banchereau et al., 2000). Endogenous DCs arise from precursors in the bone marrow, and travel through the blood to home to peripheral tissues where they dwell as iDCs with low surface expression of MHC and co-stimulatory molecules (Banchereau and Steinman, 1998). Immature DCs serve as sentinels by capturing antigens, surveying the

surrounding microenvironment (Banchereau et al., 2000). Dendritic cells can ingest antigens by fluid phase uptake or macropinocytosis and receptor-mediated endocytosis, providing them with high capacity for uptake of soluble antigens as well as specificity (Sallusto and Lanzavecchia, 1994). Dendritic cells have also been shown to internalize particles of latex (Reece et al., 2001), PLGA (Walter et al., 2001; Lutsiak et al., 2002; Newman et al., 2002), and polystyrene, depending on proteins adsorbed to particle surface (Thiele et al., 2003).

Pattern recognition receptors expressed on DCs include C-type lectins such as mannose receptor (Sallusto et al., 1995; Avrameas et al., 1996), DEC205 (Kato et al., 1998a), and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al., 2000) which bind to carbohydrate-conjugated molecules, scavenger receptors, which are involved in internalization of polyanionic ligands (Peiser et al., 2002), and Toll-like receptors (TLRs), a family of receptors whose ligation leads to upregulation of co-stimulatory molecule expression and release of pro-inflammatory cytokines and DC maturation (Medzhitov, 2001; Kaisho and Akira, 2002).

To date, more than 10 TLRs have been identified (Medzhitov et al., 1997), and it is thought that there may be 10 to 15 TLRs in the mammalian species (Iwasaki and Medzhitov, 2004). Some of the PAMPs that TLRs recognize include bacterial lipoproteins and lipoteichoic acids (TLR2), double stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), single stranded viral RNA (TLR7) and unmethylated CpG DNA (TLR9). Toll-like receptors recognize multiple PAMPs of not only bacterial and viral origin, but also molecules that may exist in the host. Strategic anatomical expression of TLRs within the host does not allow encounter of host molecules with TLRs. In this

manner, triggering of TLR activation due to recognition of host molecules is prevented. For example, TLRs 3, 7, 8, and 9 which recognize viral nucleic acids are expressed in intracellular compartments where host nucleic acids normally do not exist (Heil et al., 2003; Lee et al., 2003; Matsumoto et al., 2003). Similarly, TLR5 which recognizes flagellin is expressed on the basolateral side of the intestinal endothelial epithelia, thereby precluding encounter with commensal bacterial flagellin localized to the apical side (Gerwirtz et al., 2001). Toll-like receptors contain an extracellular leucine-rich repeat domain, a single transmembrane domain, and a cytoplasmic domain homologous to the IL-1 receptor, which is necessary for signaling (Kaisho and Akira, 2001). The cytoplasmic domain, called Toll/ IL-1 receptor homology domain, associates with an adaptor protein, myeloid differentiation factor 88 (MyD88), resulting in the activation of MAP kinase cascades and activation of nuclear factor κ B (NFκB), a transcription factor which controls the expression of many genes involved in the inflammatory response including IL-12 and TNFα (Kaisho and Akira, 2001). However, MyD88-independent pathway of TLR activation by LPS has also been observed, and can lead to DC activation (Kaisho and Akira, 2001).

While many of the C-type lectins have been implicated in endocytosis and recognition of pathogens, the presence of signaling motifs in their cytoplasmic domains suggest that C-type lectins may play a role in signaling in DCs (Figdor et al., 2002). In particular, Dectin-1, C-type lectin which recognizes β-glucans, has been shown enhance TLR2-mediated signals to activate NFκB and with TLR2, synergistically produce IL-12 (Gantner et al., 2003). Similarly, galactose/ N-acetylgalactose lectin has been observed to increase mRNA expression of TLR-2, as well as activate NFκB and MAP kinase

signaling pathways (Kammanadiminti et al., 2004). Such cooperation between lectins and TLRs suggest tailored response against specific pathogens (Gantner et al., 2003).

Maturation of dendritic cells and migration to secondary lymphoid organs

Dendritic cells mature in response to infection or "danger" signals, such as antigens from necrotic cells or pro-inflammatory cytokines (Matzinger, 1994; Banchereau et al., 2000). Maturing DCs migrate to the T cell areas of the draining lymph node, where they present the previously internalized and processed antigens in context of MHC molecules for T cell activation (Matzinger, 1994; Banchereau and Steinman, 1998; Basu et al., 2000; Bethke, 2002).

Function of DCs correlates with their migration, and the ability of DCs to migrate is dependent on the expression of adhesion molecules (Banchereau and Steinman, 1998). Migration of maturing DCs to draining lymph node via the afferent lymph is mediated by downregulation of receptor for or unresponsiveness to MIP-3α, expressed at sites of inflammation, and upregulation of expression of chemokine receptor CCR7 on DCs, whose corresponding chemokine ligands, MIP-3β and SLC are expressed by cells in the secondary lymphoid organs (Sallusto et al., 1998; Chan et al., 1999; Sozzani et al., 1999). As both MIP-3β and SLC can attract mature DCs and naïve T lymphocytes, these chemokines likely facilitate the encounter of these two cell types for the initiation of the adaptive immune response (Gunn et al., 1998; Ngo et al., 1998).

Maturation stimuli for DCs demonstrated *in vitro* include endogenous adjuvants or molecules that are produced only in the presence of "danger" to the host. Dendritic cells are activated in response to poly-mannuronic acid of pathogenic source via TLRs 2

and 4 (Flo et al., 2002). In addition, heat shock proteins (Basu et al., 2000; Bethke, 2002), and degraded proteins that only appear upon tissue injury such as oligosaccharides of hyaluronan also activate DCs via TLR 4 (Termeer et al., 2000; Johnson et al., 2002; Termeer et al., 2002). Upon maturation, DCs lose receptors for phagocytosis and endocytosis, downregulate pinocytosis, upregulate the expression of MHC and co-stimulatory molecules such as CD40, CD80, and CD86, and reorganize their cytoskeleton and increase motility (Sallusto et al., 1995; Winzler et al., 1997; Banchereau and Steinman, 1998; Banchereau et al., 2000). The antigen capturing capacity of DCs is irreversible as the removal of maturation stimulus does not result in the recovery of pinocytosis by these cells (Sallusto et al., 1995). Maturation of DCs as typified by these changes are thought to be regulated by the same signaling pathways that are activated during inflammation and cell stress, including the activation of the transcription factor, NFκB and MAP kinase, in particular, p38 and JNK (Rescigno, 1998). Translocation of NFκB from the cytosol to the nucleus upon phosphorylation of IκB for the activation of gene transcription is essential for DC maturation (Rescigno et al., 1998; Yoshimura et al., 2001). Of the 5 known members of NFκB family of transcription factors, RelB has been shown to be required for DC maturation (Wu et al., 1998; Zanetti et al., 2003), and its activity has been recently demonstrated to be negatively regulated by NFκB2, also known as p52, another member of the NFκB family (Spiers et al., 2004).

Dendritic cell subsets

Several subsets of DCs exist in various species. In humans, differences in subsets can be categorized by precursor populations, anatomical localization, function, and final outcome of the immune response (Banchereau et al., 2000). Two types of DC precursor cells in blood are myeloid monocytes (pDC1) and CD4+CD3-CD11c- plasmacytoid precursors (pDC2) (Rissoan et al., 1999). Monocytes or pDC1 can differentiate into myeloid DCs (DC1) following transmigration through the endothelium and phagocytosis of subendothelial collagen (Randolph et al., 1998) or in culture with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Romani et al., 1996), and mature in response to CD40 ligation or endotoxin stimulation (Cella et al., 1996). In contrast, pDC2 found in blood or tonsil differentiate in culture into immature DCs in presence of IL-3, and mature in response to CD40 ligand stimulation (DC2) (Grouard et al., 1997; Olweus et al., 1997). Dendritic cells derived from pDC1 and pDC2 preferentially induce Th1 or Th2 differentiation, respectively, via differential DC production of cytokines (Rissoan et al., 1999). Differentiation of Th1 is driven primarily by IL-12, where as Th2 is driven primarily by IL-4 (Abbas et al., 1996). While DC1-induced Th1 differentiation is mediated by increased production of IL-12 in response to CD40 ligand stimulation, DC2-induced Th2 differentiation is mediated by mechanisms independent of IL-4 or IL-12 (Rissoan et al., 1999).

Adjuvants

Immunological adjuvants are often incorporated in vaccines where the antigen alone is not immunogenic enough to elicit protective immunity. Adjuvants are classified by their modes of action: particulate and immunostimulatory (Singh and O'Hagan, 1999). Particulate adjuvants, including polymeric microparticles, are internalized by APCs, thereby activating them, inducing an immune response (Singh and O'Hagan, 1999). Particulate adjuvants also enhance the immune response by creating a depot of antigen at the site of injection to prolong exposure (Hunter, 2002). Complete or incomplete Freund's adjuvant (CFA/ IFA), an oil emulsion with or without killed bacteria, respectively, are commonly used adjuvants but are limited to experimental animal models due to their toxicity (Alving, 2002). To date, aluminum hydroxide, aluminum phosphate, and calcium phosphate are the only adjuvants approved for clinical use. Polymeric microparticles are currently under investigation as possible adjuvants (Ertl et al., 1996; Seferian and Martinez, 2001; Suckow et al., 2002; Jaganathan et al., 2004). Immunomodulatory adjuvants are often of pathogenic source, including bacterial DNA and lipopolysaccharides (LPS), and enhance the immune response by activating APCs through MHC and co-stimulatory molecules or by affecting cytokine release (Singh and O'Hagan, 1999). Many of these adjuvants as well as natural adjuvants such as byproducts of necrosis and mechanical stress (Gallucci et al., 1999) also activate APCs via TLRs.

Host response associated with biomaterial implants

Protein adsorption

The implantation of a medical device leads to the host response against the injury caused by the implantation procedure as well as against the material itself (Anderson, 1988). Upon introduction to the body, proteins in the plasma and interstitial fluid immediately adsorb to material surface; thus the response of the inflammatory cells to the implant is through the adsorbed proteins present on the most outer surface of the implant, directed by the biomaterial surface properties, not against the naked biomaterial surface itself (Tang and Eaton, 1995). For polymeric biomaterials, albumin, fibrinogen, fibronectin, vitronectin, immunoglobulins, and components of the complement system are commonly found to be adsorbed to the surface (Tang and Eaton, 1995). The adsorption of these proteins to biomaterial surface is influenced by surface properties such as hydrophobicity, charge, chemistry, and microtopology (Mrksich et al., 1996; Shen and Horbett, 2001; Thiele et al., 2003). Polymorphonuclear cells (PMNs) such as neutrophils and monocytes, adhere to the adsorbed proteins on material surface via their adhesion molecules and receptors to adsorbed proteins, initiating inflammation (Anderson, 1988; Tang and Eaton, 1995). In general, hydrophobic polymeric surfaces have been shown to adsorb more proteins than do hydrophilic surfaces, and hence induce increased PMN adhesion and influence cell behavior (Collier et al., 1997; Otto et al., 2003). In particular, implantation of polyester terephthalate (PET) surface pre-adsorbed with albumin has been shown to passify biomaterial surface, resulting in fewer adherent cells as compared to non-coated PET surface. In contrast, adsorption of fibrinogen to PET surface plays an important role in the inflammatory response against the implanted

PET; lack of inflammatory response against the surface in animals with low fibrinogen can be reconstituted by coating the implanted PET with fibrinogen or by injection of the animals with fibrinogen prior to implantation of non-coated PET (Tang and Eaton, 1993). In addition, fibrinogen has been shown to be the predominant component of adsorbed proteins on vascular grafts (Pankowsky et al., 1990) as well as on polymer surfaces for a variety of surface chemistry upon incubation in plasma (Nimieri et al., 1994). The role of fibrinogen in inflammatory response against biomaterials has also been corroborated by the positive relationship between monocyte adhesion to polymeric surfaces and the amount of adsorbed fibrinogen present on these surfaces (Shen and Horbett, 2001). Further, initiation of the inflammatory response has been shown to be caused by the change in the conformation of fibrinogen bound to biomaterial surface (Hu et al., 2001). A similar observation of conformational change has been observed for vitronectin using atomic force microscopy (Zhang et al., 2004).

Complement activation

For biomaterial-induced complement activation, the alternative pathway has been most characterized (Kazatchkine and Carreno, 1988), but classical pathway has also been implicated (Tengvall et al., 1996; Lhotta et al., 1998). Spontaneous binding of C3b to a material surface is covalent, and is linked by the hydroxyl or amine groups on the material surface and the carbonyl group in the thioester binding site of C3b (Gorbet and Sefton, 2004). Thus materials with nucleophiles on the surfaces promoting binding of C3b to the surface is thought to be complement-activating as this will result in the formation of C3 and C5 convertases, driving the complement activation (Gorbet and

Sefton, 2004). While mannose-binding lectin pathway of complement activation has not yet been observed in biomaterial-induced complement activation, it is probable that materials containing carbohydrates or surfaces adsorbed with proteins with exposed carbohydrate modifications may activate the complement cascade via this pathway. Other factors that influence the activation of complement pathways include the type of base polymer material, as polymers of different backbone with same surface hydroxyl group density exhibited different levels of complement activation (Labarre et al., 2002) and molecular mobility of the material substrate (Berglin et al., 2004).

Recently, the sequence of events of plasma protein adsorption and complement activation on polystyrene has been shown to occur simultaneously; while this initial activation of complements was via the classical pathway, alternative pathway of complement activation followed, resulting in the deposition of C3b atop the initially deposited layer of plasma proteins (Andersson et al., 2005). Moreover, there are interactions amongst components of the complement system and the coagulation cascade, further complicating the events at the material-tissue/ blood interface. One result of complement activation is the activation of platelets and procoagulant phenotype; however, the complement proteins that result in biomaterial-induced activation of platelets are yet to be elucidated (Gorbet and Sefton, 2004).

Because protein adsorption directs cell adhesion to material surface and subsequent cell behavior, modifications of material surface to control protein adsorption to preferentially guide cell behavior to improve biocompatibility, enhance graft integration, and induce tissue regeneration have become one of the focuses of biomaterials research. To minimize protein adsorption to material surface, grafting of

polymeric surfaces with poly(ethylene glycol) (PEG) to render the surface more hydrophilic (Noh et al., 1998) has been successful (reviewed in (Alcantar et al., 2000)). Other efforts to minimize adsorption of protein to biomaterial implants include microstructuring to change the surface wettability (Rupp et al., 2004) and plasma deposition to inhibit fibrinogen and monocyte adhesion (Shen et al., 2001). In addition, biomimetic polymers containing biological signals to promote selective host response such as macrophage behavior and tissue repair are in development (Kao and Lee, 2001; Baier Leach et al., 2003).

Acute and chronic inflammation

Following the immediate adsorption of proteins to material surface, the host response against biomaterial implants include acute and chronic inflammatory responses, granulation tissue formation, and fibrosis (Anderson, 1988; Tang and Eaton, 1995). During acute inflammation which lasts from minutes to days, phagocytes migrate to the area of inflammation mediated by adhesion molecules, where their function is phagocytosis and biochemical killing of invading pathogens by release of reactive oxygen species (Ratner et al., 1996). The recognition of the biomaterial implant by neutrophils is mediated by the opsonization of the surface by IgG, fibrinogen, or components of the complement system (Tang et al., 1993). Degree of release of reactive oxygen species has been shown, on a glass surface, to vary depending on the presence of a particular opsonins (Liu et al., 1997). Upon adhesion, neutrophils attempt to phagocytose the implant but fail due to the size of the implant, and undergo frustrated phagocytosis and release reactive oxygen species which can cause degradation of the

biomaterial (Labow et al., 2001). The extent of inflammatory response elicited by the release of reactive species by neutrophils depends on the ability of a material to be phagocytosed; large particles that cannot be phagocytosed result in increased neutrophils release of enzyme (Henson, 1971). This oxidative degradation of implanted material may not only release particles of the bulk material but also leach constituents of the material to the surrounding area, which can cause deleterious effects even at distal sites if leached systemically.

Chronic stage of inflammation is characterized by the presence of monocytes, macrophages, and lymphocytes, at as early as 1 day after the implantation (Ratner et al., 1996). Similar to neutrophils, monocytes and macrophages are also phagocytic, and may result in degradation of the implant at the material interface, leading to corrosion and generation of particulate degradation byproduct. Activation of these inflammatory cells by a biomaterial is again in part dependent on the size and the form of the implant or the likelihood of the implanted material to be phagocytosed. In particular, wear debris from the prosthetic implant are internalized by macrophages, resulting in release of inflammatory cytokines and proteolytic enzymes, leading to implant failure (Affalato et al., 2001). In contrast with the higher neutrophil release of oxidative species with large particles, culturing of these phagocytes with sterile ultra high molecular weight polyethylene particles with size and morphology similar to wear particles produced *in vivo* has shown increased cytokine production by cells cultured with particles of phagocytosable size, while cells cultured with larger particles secreted less cytokines (Matthews et al., 2000). Further, macrophages can also release chemotactic factors,

reactive oxygen species, growth factors, and cytokines to orchestrate wound healing and affect proliferation and function of multiple cell types (Anderson, 1988).

Granulation Tissue and Foreign body reaction

Release of soluble factors by macrophages which characterized the chronic inflammation lead to the proliferation of fibroblasts and endothelial cells surrounding the implant, forming granulation tissue (Ratner et al., 1996). The repair process initiated by the formation of granulation tissue may occur as soon as 3-5 days after the implantation, and is characterized by angiogenesis and migration of fibroblasts to form a loose framework of connective tissue (Ratner et al., 1996). The fibroblasts in the network originally produce proteoglycans, then collagen, which mechanically strengthen the connective tissue. Neovascularization and formation of fibrovascular tissue are differentially affected at the material/ tissue interface. In particular, surface roughness and nanostrucutures of PLGA has been shown to influence adhesion and proliferation of endothelial and vascular smooth muscle cells (Miller et al., 2004; Xu et al., 2004) and *in vivo* infiltration of fibrovascular tissue into tubes also made of PLGA was observed upon subcutaneous implantation (Day et al., 2004).

In the subsequent stages of healing, foreign body reaction as characterized by components of granulation tissue and presence of foreign body giant cells (FBGCs) ensue (Anderson, 1988). In an attempt to phagocytose the biomaterial implant, monocytes and macrophages adhere to the implant and fuse, forming FBGCs (Ratner et al., 1996). This cellular adhesion to biomaterial and subsequent fusion of macrophages are influenced at least in part by surface properties (Shen and Horbett, 2001; Collier et al., 2004; Dadsetan

et al., 2004), and the consequent adsorbed proteins and their orientation (Kao et al., 1999; Collier and Anderson, 2002), as well as the cytokine environment surrounding the biomaterial implant (Kao et al., 1995). The degree of foreign body reaction is influenced by the surface roughness of the biomaterial; foreign body reaction on smooth surfaces are characterized by the presence of macrophages of one to two cells in thickness, while rough surfaces primarily present foreign body reactions composed of macrophages and FBGCs (Anderson, 1988). Formation of FBGCs *in vitro* is induced by IL-4 (McInnes and Rennick, 1988) and IL-13 (DeFife et al., 1997). In experimentally induced *in vitro* FBGC formation by IL-4, the initial adhesion of monocytes is mediated by β_2 integrins, whereas the fusion of the macrophages is mediated by β_1 (McNally and Anderson, 2002). In addition, mannose receptor, whose expression is increased upon IL-4 or IL-13 addition, also plays a role in the fusion of macrophages (McNally et al., 1996; DeFife et al., 1997). More recently, subcutaneous implantation of biomaterial in animals lacking CC chemokine ligand, CCL2/ MCP1 has been shown to have altered macrophage fusion and reduced FBGC formation, implicating a role for CCL2/ MCP1 in the fusion of macrophages for the formation of FBGCs (Kyriakides et al., 2004). It is of particular interest to note that the *in vitro* methods for the generation of FBGC formation from peripheral blood mononuclear cells, either with GM-CSF and IL-4 (Kyriakides et al., 2004) or IL-4 alone (Dadsetan et al., 2004) are very similar to those used to induce formation of DCs *in vitro* (Romani et al., 1996) as used in this research.

Fibrosis

Development of fibrous capsule around the biomaterial to wall off the implant marks the later stages of wound healing (Tang and Eaton, 1995; Ratner et al., 1996). While in an ideal restoration, tissue surrounding the implant would be regenerated by parenchymal cells, but replacement of tissue by fibrotic capsule and connective tissue is more common (Anderson, 1988; Tang and Eaton, 1995). In the case of organ substitutes, the generation of fibrotic overgrowth, especially if not well vascularized, impedes exchange of nutrients and waste between the microenvironment and the cells, and is to be minimized. As with many other aspects of host response against biomaterial implants, modification of biomaterial surface to control fibrosis around implants include modification of surface with biomimetic peptide (Johnson et al., 1997), PEG (Sawhney et al., 1993), and phosphorylcholine (Malik et al., 2001).

Other cellular responses to biomaterials include the upregulation of heat shock proteins (HSPs), of which HSP 70 and 90 are the most studied (Kato et al., 1998b; Santoro, 2000; Weber et al., 2001; Parcellier et al., 2003). The expression of some HSP is constitutive, while other HSP may be induced by heat shock and other cytotoxic conditions. Heat shock proteins protect the cells, often via their function as molecular chaperones, by stabilizing the folding of proteins damaged due to stress (Santoro, 2000; Weber et al., 2001; Parcellier et al., 2003). The differential mRNA expression of some HSPs by several cell types in contact with various biomaterials (Kato et al., 1998b; Klinge et al., 2002; Wang et al., 2003) suggest that contact with polymer may provoke cellular stress.

Biomaterials in combination products

Various tissue engineering strategies such as immunoisolation and 3-dimensional constructs composed of a carrier matrix or scaffold seeded with viable functional cells have shown promise in reparative medicine (Langer and Vacanti, 1993). The biomaterial component of the device serves not only as a scaffold for the cells but also guides cell growth, maintenance of differentiation state, tissue regeneration, and vascularization, especially with the addition of growth factors (Chapekar, 2000; Langer, 2000; Humatcher, 2001). To achieve these functions, a biomaterial must be biocompatible and possess controllable microstructure, degradability, porosity, and mechanical and physical strength and integrity. Moreover, these biomaterials must be readily available, reproducible, processable, and withstand appropriate sterilization processes for clinical applications (Marler et al., 1998; Kim et al., 2000; Langer, 2000).

Both natural and synthetic biomaterials have been explored for use in tissue engineering. Biomaterials of natural origin are advantageous in that they may contain amino acid sequences that promote desirable cellular function, but may face difficulties in production including reproducibility between batches and scale-up (Langer and Vacanti, 1993; Angelova and Hunkeler, 1999; Kim et al., 2000). Major advantage of synthetic biomaterials is the ability to control physicochemical properties including molecular weight, hydrophobicity, composition, and degradability; however, synthetic materials do not have intrinsic biological recognition and may be less biocompatible compared to the naturally occurring materials (Angelova and Hunkeler, 1999; Kim et al., 2000; Langer, 2000).

In other combination product application such as drug delivery, various biomaterials have been explored. In particular, micro- and nanoparticles of biomaterials as adjuvants is an attractive approach as particle internalization can target the delivery of the associated antigen to APCs. In addition, control of polymer degradation rate enables controlled release, making polymer particles an ideal vehicle for drug delivery (Jiang et al., 2005). To this end, several natural and synthetic materials with immunomodulating properties including PLGA, chitosan, and ethylene imine have been investigated (Storni et al., 2005). For induction of mucosal immunity, properties of biomaterials that promote adhesion to mucous membranes such as charge can enhance the mucosal protective immunity via uptake of the antigens by M cells, specialized APCs in the mucosa, resulting in production of IgA (Kuntz and Saltzman, 1997; Brayden and Baird, 2004).

Poly(lactic-*co*-glycolic acid)

Poly(lactic-*co*-glycolic acid) (PLGA) is synthetic polyester composed of variable molar ratios of lactic and glycolic acid. It is biodegradable via bulk hydrolysis and biocompatible as its degradation products, lactic and glycolic acids, can be metabolized and excreted (Athanasiou et al., 1996). Rate of PLGA hydrolysis can be controlled by its geometric size, ratio of lactic to glycolic acid, and molecular weight of the polymer (Hutchinson and Furr, 1986; Eldridge et al., 1991; Mikos and Temenoff, 2000). For drug delivery applications, hydrolysis rate can be exploited for controlled release (Newman et al., 1998). The immune response supported by PLGA as an adjuvant, as measured by antibody responses against antigens delivered with PLGA, has been evaluated as at least comparable if not greater than that elicited by known adjuvants such as alum and

Complete Freund's Adjuvant (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001). Micro- and nanoparticles of PLGA have also been shown to elicit cell-mediated immune response which cannot be induced by alum (Newman et al., 1998). Furthermore, particle size and the mode of antigen association with the polymer (adsorption or entrapment) affect the immunogenicity of PLGA (O'Hagan et al., 1993), presumably due to differential phagocytosis by APCs. Microspheres of PLGA have been shown to be phagocytosed by macrophages and DCs, and retained in the phagosomal vesicle (Walker et al., 1998). Recently, studies investigating the effects of PLGA microspheres as drug delivery vehicles and their uptake by DCs have been reported. In the murine system, PLGA microspheres were shown to enhance antigen presentation by DCs (Sun et al., 2003), and clinical grade human DCs generated in the absence of plasma or serum were able to ingest PLGA microspheres without changing surface expression of co-stimulatory and MHC molecules (Waeckerle-Men et al., 2004).

Because of the biodegradability of PLGA, and the ability to control this degradation, as well as its mechanical strength, this polymer has also been extensively researched for tissue engineering applications (Jain, 2000). Adjuvant effect of PLGA in the context of tissue engineering has been shown; delivery of PLGA microparticles or scaffolds by injection or implantation, respectively, with a model shed antigen has resulted in increased production of antigen-specific antibody (Matzelle and Babensee, 2004), and this increased humoral response was dependent on the form of PLGA (Bennewitz and Babensee, 2005). As materials such as PLGA is explored for both vaccine delivery and tissue engineering applications where adjuvant effect and associated increase in immunity is or is not desirable, respectively, understanding of biomaterial

adjuvant effect is paramount to the development of materials for use in combination products.

Agarose

Agarose is a polysaccharide isolated from seaweed, composed of repeating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl units. It gels thermoreversibly due to hydrogen bond formation, facilitated by alignment of the agarobiose molecules (Shoichet et al., 1996). Agarose has been commonly used as a food ingredient stabilizer, and as a biomaterial for tissue engineering, including 3-D culture of chondrocytes (Benya and Shaffer, 1982; Sun et al., 1986), neural cells and nerve regeneration (Bellamkonda et al., 1995), and immunoisolation of islets for bioartificial pancreas (Iwata et al., 1992). Host response against implanted agarose has been studied in several applications. Injection of sterile agarose beads into the trachea of mice resulted in minimal local inflammatory reaction (Starke et al., 1987), and bronchoalveolar lavages from mice inoculated with sterile agarose beads contained low levels of inflammatory cytokines with little cellular response, which consisted of mononuclear cell infiltration only around the beads (van Heeckeren et al., 2000). In another application, animals receiving agarose gel alone into created defects penetrating the cartilage showed no immune cell infiltration or inflammation in the synovium after 12 months (Rahfoth et al., 1998), demonstrating low immunogenicity of agarose. Agarose has also been used as carrier vehicle for the delivery of cytokines for wound healing (Rollwagen et al., 1993; Spargo et al., 1994) as well as for corticosteroid-producing adrenal cells for reduction of inflammation (Cadic-Amadeuf et al., 1992). While these studies vary in the physical properties of agarose

used, the concentration of agarose and the site of implantation, they collectively suggest that agarose elicits minimal humoral and cellular response *in vivo*.

Summary

In efforts to improve compatibility of biomaterials used in combination products and implants, the host response to biomaterials has been studied at various phases, from the initial stages of protein adsorption to the resulting consequences on the adaptive immunity. Despite progress in understanding the effect of biomaterials alone on the immune and inflammatory responses, particular concern remains in the case of combination products comprised of biological and biomaterial components. When introduced to the host, inflammatory response against the biomaterial and immune response against the biological or cellular component may act in concert, leading to deleterious effects. The presence of biomaterial can enhance the adaptive immune response through its adjuvant effect mediated by the maturation of DCs. While many of the studies of immune and inflammatory responses in the context of biomaterials to date have focused on these responses separately, the understanding of the link between how the innate response of inflammation against biomaterials interact with and influence the adaptive immunity remains largely unexplored.

CHAPTER 4

CHARACTERIZATION OF MICROPARTICLES AND FILMS OF POLY(LACTIC-*CO*-GLYCOLIC ACID) AND AGAROSE

INTRODUCTION:

The use of biomaterials in biomedical applications in which the biomaterial together with biological components encounter the host defense mechanism necessitates the clarification for the role of the biomaterial in supporting or not supporting the immune response associated with the biological component. In particular, for biomaterials used in tissue engineered devices and combination products where the biomaterial serves both structural and functional purposes, the interaction between inflammatory and immune responses elicited by the biomaterial and the biological components, respectively, may result in unexpected detrimental effects when concurrently imposed (Babensee et al., 1998). To understand the adjuvant effect of the biomaterial in potentiating the immunological response against the biological component, the effect of biomaterial contact on dendritic cell maturation was investigated. In particular, microparticles (MPs) and films of two model biomaterials currently explored in these applications, poly(lactic-*co*-glycolic acid) (PLGA) and agarose, were prepared and characterized for subsequent testing of their adjuvant effect by measuring the extent of dendritic cell maturation induced. While the characterizations of these biomaterials are not novel, it is of importance, especially as most MPs and films were made in-house, to fully understand the effect of the biomaterial and its properties on dendritic cell maturation.

METHODS:

Preparation of poly(lactic-co-glycolic acid) microparticles

Poly(lactic-*co*-glycolic acid) MPs were prepared by a single emulsion solvent evaporation technique adapted from a previously described method (Wake et al., 1998). Briefly, for small MPs (~3 μm diameter) 500 mg of PLGA (molar ratio: 75:25, inherent viscosity: 0.69 dL/g in trichloromethane) (Birmingham Polymers, Birmingham, AL) was dissolved in 20 ml dichloromethane (DCM) (Sigma, St. Louis, MO) overnight at room temperature. On the second day, the PLGA-DCM solution was added to 200 ml 0.3% v/v aqueous poly(vinyl alcohol) (88% hydrolyzed) (PVA) (Sigma), and homogenized for 2 min at approximately 9000 rpm using a PowerGen Homogenizer 700 (Fisher Scientific, Pittsburgh, PA). Immediately after homogenization, 200 ml 2% (v/v) aqueous isopropanol (Sigma) was added to initiate precipitation, and the mixture stirred overnight at room temperature to evaporate the solvent. Resulting MPs were collected by centrifugation at 1000 rpm for 10 minutes, washed twice in 2% isopropanol, and three times in distilled deionized H_2O (ddH_2O). Microparticles were resuspended in ddH_2O and their size distribution characterized using a Coulter Multisizer II (Coulter Corporation, Miami, FL). To make larger MPs (~30 μm diameter), same procedures as described above were used, with an exception of a lower homogenization speed of 950 rpm. Resulting MP suspension was collected by centrifugation 1000 rpm for 5 minutes, then washed twice in 2% isopropanol. Upon washing with isopropanol, the MPs were successively filtered using a 70 μm cell strainer, then through a 40 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Resulting filtrate was filtered again using a 20 μm nylon mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA). This time the

MPs in the membrane retentate were collected by washing the mesh with at least 50 ml ddH_2O. The resulting MP suspension was washed 3 times with ddH_2O. For both size MPs, MP suspension was UV sterilized for 1 hr, and stored at 4°C for no longer than 3 days prior to use.

Preparation of poly(lactic-co-glycolic acid) film

Poly(lactic-*co*-glycolic acid) films were fabricated using a previously described casting technique without a porogen (Ishaug et al., 1997). Briefly, 10% w/v 75:25 PLGA was dissolved in DCM overnight at room temperature. The PLGA-DCM solution was poured onto a 50 mm or 100 mm Teflon Petri dish (Cole-Parmer, Vernon Hills, Illinois). Upon evaporation of the solvent and drying, the film was punched into an appropriate size and washed for 1 hr in ddH_2O, changing water every 15 min. The film was dried 30 min per side in a sterile laminar flow hood and UV sterilized for 30 min per side prior to use.

Preparation of agarose microparticles

Microparticles of polystyrene coated with agarose (agarose MPs), of 2 μm and 30 μm in diameter, were purchased from Micromod (Micromod, Rostock-Warnemuende, Germany). Prior to use, the MPs were washed three times in 70% ethanol and three times in ddH_2O.

Preparation of agarose film

Agarose (type V; sulfate content of ≤ 0.30%, gel strength of ≥800 g/ cm^2 at 1.0%, and gel point of 42°C±1.5°C at 1.5%) was purchased from Sigma. This particular agarose was chosen as it had a high gel point, enabling it to remain in the hydrated gelled state at temperature of 37°C, temperature used for cell culture. Aqueous solution of 3% w/v agarose in ddH_2O was prepared and heated using a microwave until boiling and visible homogeneity was reached. The film was prepared by dispensing 1 ml of agarose solution into a well of a 6 well plate (Corning, Corning, NY), and allowed to solidify at a temperature of 4°C for at least 30 min, and brought back to room temperature for another 30 min prior to use.

Scanning electron microscopy (SEM)

Morphology of MP surface was analyzed by scanning electron microscopy (SEM) at Integrated Microscopy and Microanalytical Facility at Emory University. Samples of agarose or PLGA MPs were prepared by placing a drop of MP suspension on a silicon chip and lyophilized. Sample of PLGA film was prepared by mounting a piece of excised film on a silicon chip. Silicon chip with lyophilized MPs or PLGA film was mounted onto an aluminum specimen stub with silver paste, degassed for 30 min, and coated with 1 nm chromium using a turbo-pumped sputter system (Denton DV-602, Denton Vacuum, Moorestown, NJ). Micrographs were captured using ISI DS-130 Schottky Field Emission SEM (Topcon, Paramus, NJ). Two independent specimens were visualized.

For agarose film, cryo-high resolution SEM (HRSEM) was performed to image the film in the hydrated state. Small piece of agarose film was excised and fit into a gold planchette, and plunge frozen in liquid ethane at -183°C to form vitreous ice, and immersed in liquid nitrogen to maintain temperature below -170°C. The specimen was then placed onto a Gatan 3500 CT cryostage precooled to <-170°C and the surface of the specimen fractured with a pre-cooled blade. For some samples, the specimen surface was etched by raising the temperature from -170°C to -125°C or -105°C for 5 minutes, to remove surface exposed ice by sublimation. Samples were coated in a Denton DV-602 chromium coater (Denton, Moorestown, NJ), at a pressure of $2x10^{-7}$ torr, for a final coating of 2 nm in thickness. After coating, the specimen was transferred to a DS-130F field emission scanning electron microscope (Topcon), and images recorded.

X-Ray photoelectron spectroscopy (XPS)

Chemical compositions of biomaterial surfaces were analyzed by XPS at Georgia Institute Technology Microelectronics Research Center. Spectra of biomaterial MPs and films were obtained on a Surface Science Laboratories X-100 spectrometer (Surface Sciences Laboratories, Mountain View, CA) with monochromatized A1 *Kα* X rays using 10 kV. For MPs, specimens were prepared by adding a drop of MP suspension to a glass cover slip, dried overnight, resulting in a mound of MPs, which was then lyophilized (Shakesheff et al., 1997). Specimens were placed under a nickel mesh, and 5 eV flood gun was used to assist with the compensation for differential charging. Atomic percentages of elements were derived from low resolution spectra (spot size 400 μm). High resolution C1*s* spectra (spot size 200 μm) were obtained and resolved using curve

fitting routines provided by the manufacturer, and the binding energy scale was adjusted to place the hydrocarbon peak at 284.6 eV. For angle resolved XPS, the takeoff angle (defined as the angle between the beam and the surface normal) was varied to allow measurements at different depths from the film surface. The limits of detection of angle resolved XPS is approximately 0.1 atom % in concentration and 10-250Å in depth (Ratner et al., 1996), with maximum depth achieved at a takeoff angle of 0°.

Advancing water contact angle measurements

Ambient air-water-substrate advancing contact angles for agarose and PLGA films were measured using a Ramé-Hart goniometer (Ramé-Hart Instrument Co., Mountain Lakes, NJ) in Dr. Andrés García's laboratory at Georgia Institute of Technology with technical assistance from Dr. Ben Keselowsky. To ensure flat surface, PLGA film was prepared by pouring PLGA-DCM solution onto a cleaned cover slip placed in Teflon Petri dish. Upon polymerization as described in a previous section, excess film around the cover slip was excised. For agarose specimens, 3% agarose solution was dispensed onto a cleaned glass slide, and after solidification, the film was peeled off. The side exposed to the glass slide was used for contact angle measurements to ensure a flat surface, as the side exposed to the air during solidification was domed due to surface tension. Values reported represent the average and standard deviation of three measurements taken from each of 2 samples for each surface for a total of 6 measurements.

Gas chromatography-mass spectrometry (GC-MS)

Electron ionization GC-MS was carried out at Georgia Institute of Technology Mass Spectrometry laboratory using VG Instruments 70SE (ThermoElectron Corporation, San Jose, CA) interfaced to a Hewlett-Packard 5890 gas chromatograph (Agilent Technologies, Palo Alto, CA) in the electron ionization mode. The electron energy was 70 eV, and the mass resolution was 1000. Spectra were recorded over a range of probe temperatures, as increasing temperatures facilitates fragmentation of the compound.

RESULTS:

Characterization of PLGA microparticles

Morphology of the PLGA MPs was visualized by SEM. Both sizes of PLGA MPs had a smooth surface, with occasional pits (Figures 4-1a, b). Size distribution of the MPs as determined by SEM micrographs was in agreement with those determined by a Coulter Counter, either ~ 3 or ~ 30 μm. Results of surface chemical compositions are summarized in Table 1, and corresponding XPS spectra are shown in Figure 4-2. For both PLGA crystals and PVA powder specimens, experimental values of percentage of carbons bonded in C-C form were higher than theoretical values (Table 1, Figures 4-2a and 4-2b, respectively). Further, PLGA MPs contained higher amounts of C-C bonded carbons as compared to PLGA crystals (Table 1). No residual DCM was detected within the depths analyzed by XPS. In addition, DCM was not detected by GC-MS, as indicated by the lack of base or M+ peaks at mass: charge ratios (m/z) of 49 and 84, respectively (Figures 4-3a, b).

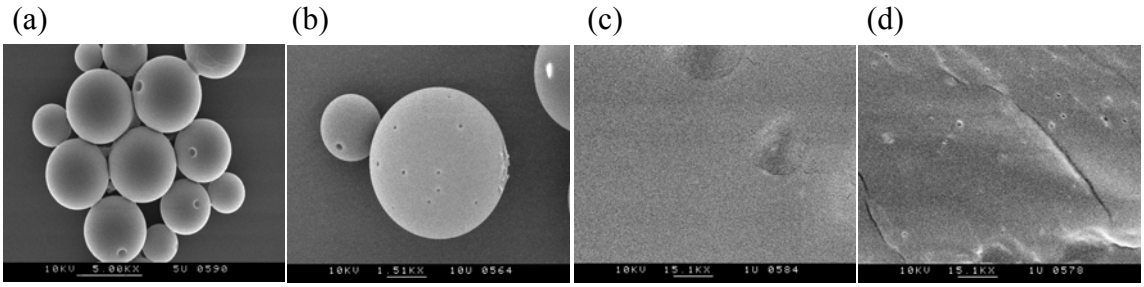

Figure 4-1: Scanning electron micrographs of PLGA MPs (3 μm (5,000x) (a), 30 μm (1,500x) (b)) and films (top surface (15,100x) (c), bottom surface (15,100x) (d)). Microparticles of both sizes show smooth surface with occasional pits. Top surface of PLGA films present smoother surface as compared to the bottom surface.

Table 1: Elemental composition in the C1*s* region of XPS spectra at 55° take off angle and advancing water contact angle for PLGA

Material	C-C	C-O	O-C=O	contact angle
PLGA crystals (theoretical)	24.7%	37.3%	37.3%	
PLGA crystals (experimental)	34.6%	30.4%	35.0%	
PVA powder (theroretical)	50.0%	44.6%	5.4%	
PVA powder (experimental)	54.6%	40.5%	4.9%	
PLGA MPs (experimental)	65.9%	20.9%	13.2%	
PLGA film (experimental)	27.4%	30.4%	42.3%	71.6° ± 2.0°

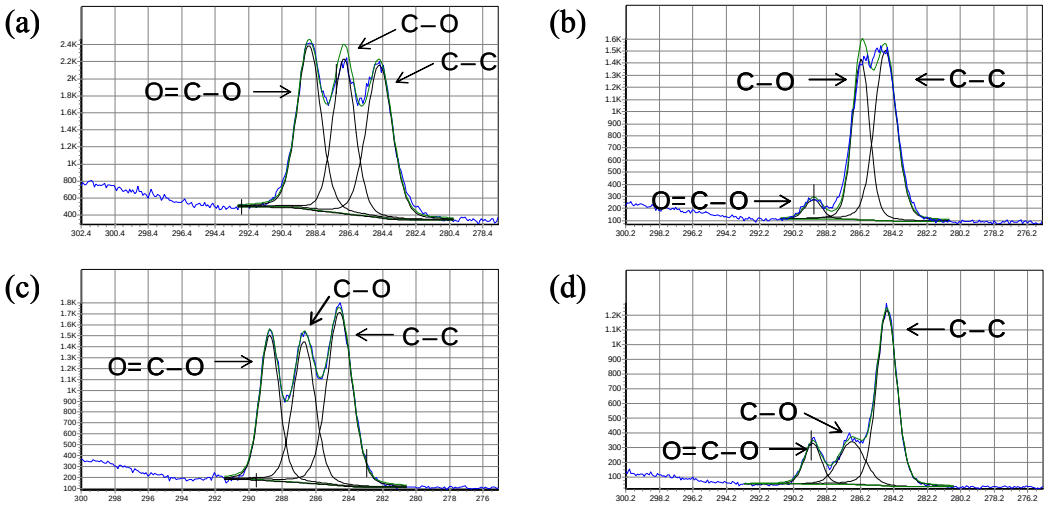

Figure 4-2: XPS spectra of PLGA crystals (a), PVA powder (b), PLGA film (c), and PLGA MPs (d).

Jagged lines represent original XPS experimental data, thin smooth lines represent calculated curve from curve fitting; the curve from original XPS data may be obscured by the calculated curve when there is a good fit. Bold smooth curves represent individual carbon environments from curve fitting as indicated.

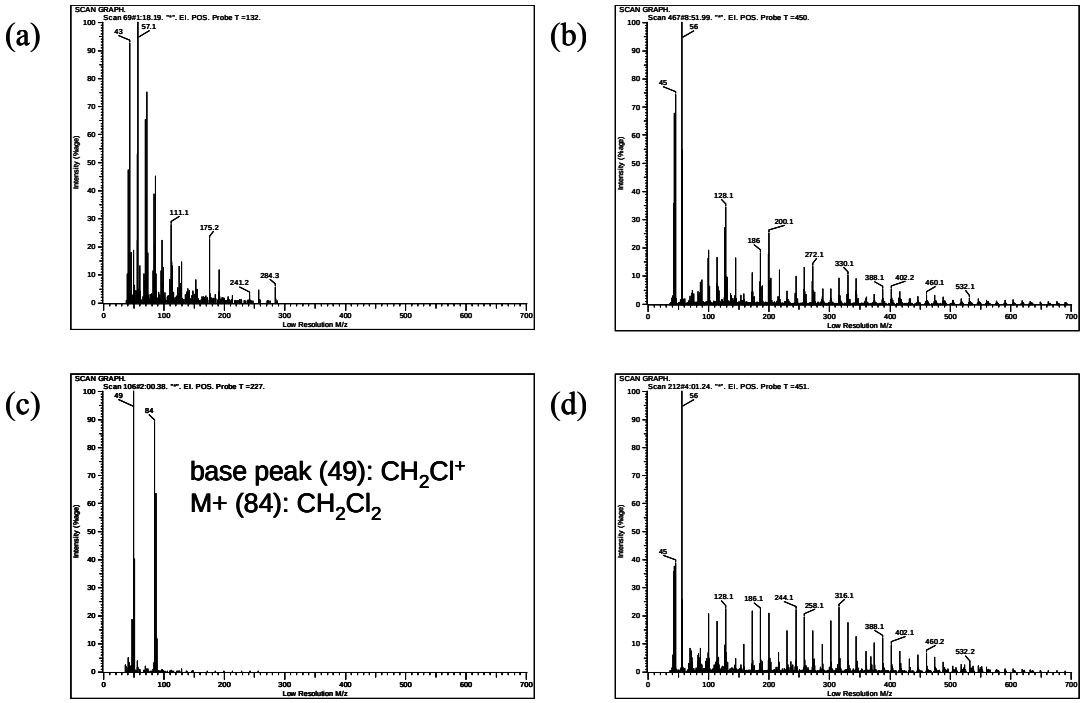

Figure 4-3: GC-MS measurements of PLGA MPs and PLGA film.
PLGA MP analyzed with probe temperature at 132°C (a) and 450°C (b). PLGA film analyzed with probe temperature at 227°C (c) and 451°C (d).

Characterization of PLGA films

Surface morphology of PLGA films as determined by SEM showed a smooth surface. In particular, the top surface (Figure 4-1c), which was exposed to the air during polymerization, was smoother than the bottom surface (Figure 4-1d), which was exposed to the casting Teflon dish. The chemical composition of the surface is summarized in Table 1, and XPS spectra of C1*s* scan is shown in Figure 4-2d. Unlike the MPs, PLGA film presented lower amounts of carbon bonded in C-C form, and increased C-O and C=O forms, a profile more like the PLGA crystals. Depth profile of chemical composition of PLGA film was measured by varying the take off angles of XPS. Throughout the depths examined, no chlorine was present. In addition, these values indicated that the ratio of C:O was higher than expected theoretical values, and increased at depths closer to the exposed surface (Table 3). While no chlorine was detected within the analytical sensitivity of XPS, GC-MS results revealed presence of residual chlorine from DCM, especially at the low probe temperature of 227°C, as indicated by peaks at m/z= 49 and 84 (Figure 4-3c). Advancing contact angle measurements for air-water-PLGA was 71.6°±2°, indicating hydrophobicity (Table 2), and in agreement with previously reported values (Karp et al., 2003).

Characterization of agarose microparticles

Agarose microspheres of both sizes presented relatively smooth surfaces with some irregularities (Figures 4-4a and 4-4b, respectively). On the surface of the 2 μm MPs, the type of carbon present was all singly bonded to oxygen (Table 2, Figure 4-5c). However, in contrast, 30 μm MPs showed lower levels of carbon bonded in the C-O form and higher levels in the C-C form (Figure 4-5d). Although agarose microspheres were submitted for analysis by matrix assisted laser desorption/ ionization (MALDI) mass spectrometry, no spectrum was obtained due to high molecular weight and polydispersity index than what can be resolved using this technique.

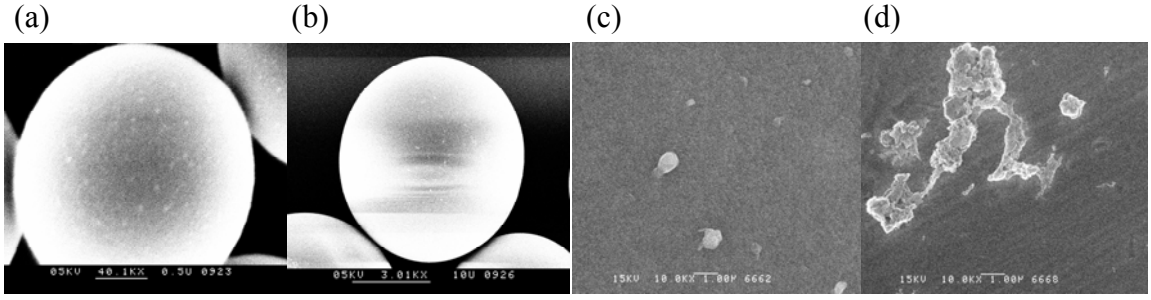

Figure 4-4: Scanning electron micrographs of agarose MPs (2 μm (40,100x) (a), 30 μm (3,010x) (b)) and agarose film (etched (10,000x) (c), non-etched (10,000x) (d)). Microparticles of both sizes show smooth surface with some irregularities (2a, 2b). Film surfaces, etched (c) and non-etched (d), are smooth with some protrusions.

Characterization of agarose films

Surface morphology of agarose film was imaged using cryo-HRSEM to preserve the hydrated state of the film. The film surfaces of both etched and non-etched presented some irregular protruding structures (Figures 4-4c and 4-4d, respectively). Surface chemical compositions analyzed by XPS revealed that all of the carbon present on the film surface was bonded in the C-O form (Table 2, Figure 4-5b), and similar to PLGA film, with decreasing depth, increased C:O ratio was observed (Table 3). Advancing contact angle measurement for agarose film was not possible as the surface of film wetted when contacted with a drop of ddH_2O indicating hydrophilicity. No successful spectrums were obtained using MALDI.

Table 2: Elemental composition in the C1*s* region of XPS spectra at 55° take off angle and advancing water contact angle for agarose

Material	C-C	C-O	O-C=O	contact angle
agarose powder (theoretical)	0.0%	100.0%	0.0%	
agarose powder (experimental)	0.0%	100.0%	0.0%	
agarose MPs (experimental)	0.0%	100.0%	0.0%	
agarose film (experimental)	0.0%	100.0%	0.0%	surface wets

Table 3: Variation in C:O ratios for PLGA and agarose films as a function of XPS take off angle

take off angle	PLGA film	agarose film
0°	75:25	54:45
41°	81:19	56:44
60°	82:18	57:43
75°	90:10	59:41

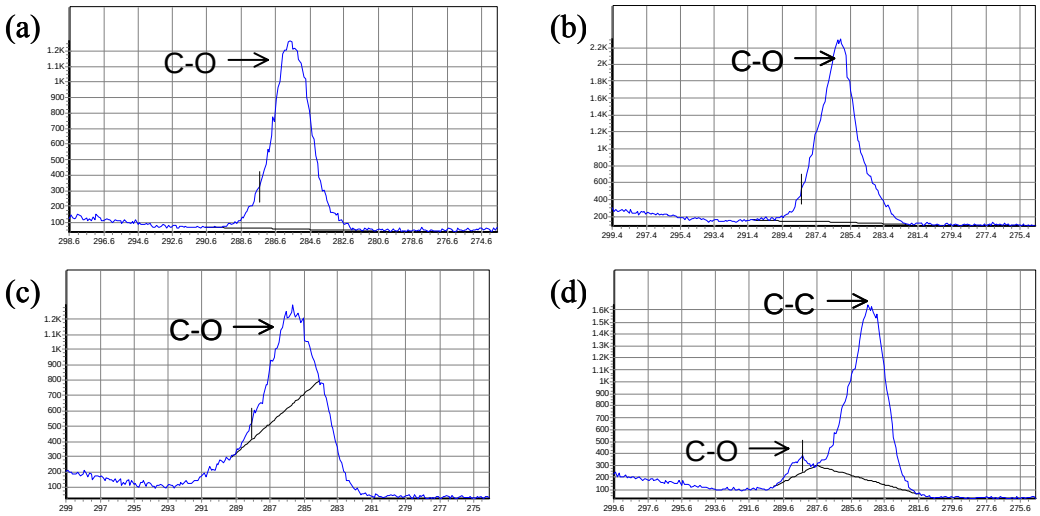

Figure 4-5: XPS spectra of agarose powder (a), agarose film (b), 2 μm MPs (c), and 30 μm MPs (d).
Jagged lines represent original XPS experimental data, thin smooth lines represent calculated curve from curve fitting; the curve from original XPS data may be obscured by the calculated curve when there is a good fit. Bold smooth curves represent individual carbon environments from curve fitting as indicated.

DISCUSSION:

Characterizations of PLGA and agarose MPs and films prepared in our laboratory for treatment of dendritic cells are reported herein. These biomaterials, in MP or film form, are cultured with dendritic cells to evaluate their associated adjuvant effect by measuring the extent of dendritic cell maturation induced upon contact with the material. Thus while characterizations of PLGA and agarose have been reported elsewhere, this assessment serves as a basis for the subsequent work presented.

Scanning electron microscopy revealed smooth surface on both PLGA films and MPs, in accord with other studies (Feng and Huang, 2001; Liu et al., 2003; Bouissou et al., 2004; Croll et al., 2004). The experimental values of chemical composition of PLGA MPs as determined by XPS showed higher amounts of carbon bonded in the C-C form as compared to theoretical values for PLGA crystals (Table 1, Figure 4-2d). This may be attributed to residual PVA, a surfactant used in the emulsification process in the fabrication of PLGA MPs. While the exact amount of residual PVA on PLGA MPs was not quantified in this study and is difficult to estimate, it has been shown that particles with higher amounts of surface PVA are phagocytosed at a lower level, perhaps due to increased surface hydrophilicity at the surface (Meese et al., 2002; Sahoo et al., 2002). Residual chlorine from DCM was not detected on PLGA MPs as measured by XPS or GC-MS. The surface of PLGA film showed chemical composition similar to that expected for PLGA crystals (Table 1, Figure 4-2c), and similar to values reported by others (Croll et al., 2004). Unlike with the PLGA MPs, the fabrication procedure for PLGA film does not require any additional agents other than the polymer itself and the solvent, DCM, which may have resulted in its chemical composition more closely

resembling that of PLGA crystals than did the PLGA MPs. Angle resolved XPS was performed on PLGA film to evaluate the presence of DCM at various depths. Within the analytical sensitivity/ detection limits of XPS of 250Å in depth and 0.1 atom % (Ratner et al., 1996), DCM was not detected, as determined by the absence of chlorine (Table 3). However, DCM was detected by GC-MS (Figures 4-3c, d), suggesting that the DCM was present in the film, at depths deeper than was reachable by XPS analysis. Angle resolved XPS also revealed an increase in the ratio of C:O closer to the surface, a phenomenon also observed by others, perhaps due to hydrocarbon contamination from the sample introduction, nearer the surface (Meese et al., 2002). Advancing water contact measurements on PLGA film showed hydrophobic surface, in agreement with previously reported values (Meese et al., 2002; Karp et al., 2003).

Scanning electron micrographs of agarose MPs presented surface with relatively smooth features with some irregularities (Figures 4-4a, 4b). These irregularities may be due to the coating, as these microspheres are of polystyrene base, covalently coated with agarose per proprietary methods by the manufacturer. Cryo-HRSEM analysis of agarose film showed that the surface of agarose film is also flat with some occasional protrusions, similar to what has been previously reported, using cryo-HRSEM, for the surface of frozen double distilled water (ddH_2O) under high pressure (Menger et al., 2004). The resemblance of surface structure of agarose film to ddH_2O is not surprising as the agarose film is composed of 97% ddH_2O and only 3% agarose. The chemical analysis of agarose film surface by XPS high resolution C1*s* scan showed that for MPs of both sizes, only 1 peak corresponding to C-O was present, in accord with theoretical values (Figures 4-5a, b, Table 2). However, while 2 μm MPs showed composition as expected of agarose

(Figure 4-5c), 30 μm MPs had some carbon bonding in the C-C form (Figure 4-5d). This increase in C-C environment may be attributed to exposure of polystyrene base due to reduced coverage of the MPs by agarose. Similar to PLGA film, C:O ratio of agarose film also increased with decreasing depth from the film surface, as measured by angle resolved XPS. Water contact angle measurements on agarose film were not possible as the surface wetted when contacted with a drop of water indicating hydrophilicity. This hydrophilicity of agarose film is not surprising as the film is composed of 3% (w/w) agarose in water.

Characterization of biomaterials to be tested for adjuvant effect via dendritic cell maturation, namely agarose and PLGA MPs and films, are reported. The increased use of these two particular biomaterials in combination products and tissue engineering applications implies a need to fully understand the material itself and their effects on the host immune system. Especially as most of the MPs and films to be tested are made in our laboratory, while utmost care is taken into their fabrication, quality control for these MPs and films may not be as regulated as for commercially available products. In subsequent studies evaluating the effect of biomaterial on dendritic cell maturation, MPs and films as reported here were used.

CHAPTER 5

POLY(LACTIC-*CO*-GLYCOLIC ACID) MODERATELY ENHANCES MATURATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS*

INTRODUCTION:

Tissue engineering has become an attractive approach for the development of functional tissue or organ substitutes where no suitable alternative exists or to reduce morbidity associated with current procedures. Success of such engineered constructs depends on the viability of functional cells and the physiological integration of the device into the host living system. As such, implanted tissue engineered devices must not only be accepted by the host immune system but also elicit minimal inflammation to avoid replacement of the seeded cells with or formation of excessive fibrotic or granulation tissue. The response against an implanted tissue engineered device may be comprised of a specific immune response against the cellular component, similar to transplanted organ allograft or xenograft rejection depending on the cell source, as well as a nonspecific inflammatory response against the biomaterial component of the device (Babensee et al., 1998). While encapsulation and other immunoisolation strategies were originally developed to prevent the detection of the implanted cells by host immune surveillance, antigens or other cellular components shed or secreted from the implanted cells may escape through the membrane, resulting in indirect recognition by the host immune cells and immune stimulation (Babensee and Sefton, 2000). This recognition and the ensuing immune stimulation may be intensified by the presence of a biomaterial component through induction of an innate immune response, recruiting antigen presenting cells (e.g.,

* Modified from Yoshida M, Babensee JE. Poly(lactic-*co*-glycolic acid) enhances maturation of human monocyte-derived dendritic cells. J Biomed Mater Res A. 2004 Oct 1;71A(1):45-54.

macrophages and dendritic cells) and inducing their activation which would lead to the activation enhancement of the adaptive response (Matzelle and Babensee, 2004). In this way, the biomaterial component can act as an adjuvant in the immune response towards associated shed cell antigens from a tissue engineered construct (Matzelle and Babensee, 2004). The elevation of specific immune response against the antigens associated with the device will lead to compromised effectiveness or failure of the device (Babensee et al., 1998). While a polymer often used in tissue engineering, poly(lactic-*co*-glycolic acid) (PLGA), acted as an adjuvant in the enhancement of the humoral response against a co-delivered model antigen (Matzelle and Babensee, 2004), an understanding of the mechanism of this observed biomaterial adjuvant effect, especially on the cellular level, remains largely unexplored.

The basis for the action of adjuvants is the role of innate immunity in stimulating adaptive immune responses. Dendritic cells recognize pathogens through conserved structures, uniquely characteristic of microbial pathogens, through their cognate binding receptors resulting in their maturation such that they become efficient antigen presenting cells (Janeway and Medzhitov, 1998). For example, constituents of mycobacteria, the active component of the strong adjuvant complete Freund's adjuvant (CFA), induce the maturation of DCs as exemplified by induction of pro-inflammatory cytokine secretion (e.g., IL-12) and upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules (Tsuji et al., 2000). Concomitant with these characteristics of mature DCs is the prolonged expression of antigen-MHC on the surface of DCs, again supporting the stimulation of T cells (Cella et al., 1997). Adjuvants may also function through the prolonged retention of the antigen in the vicinity of the inflammatory

stimulus – the depot effect – typical of controlled release devices for antigen delivery for vaccines (Zhao and Leong, 1996).

Dendritic cells play a central role in bridging the innate and adaptive immunity. Dendritic cells are professional antigen presenting cells capable of initiating an adaptive response (Banchereau and Steinman, 1998). Immature DCs efficiently serve as sentinels surveying the surrounding microenvironment by capturing and internalizing antigens (Banchereau et al., 2000). These DCs have also been shown to internalize particles of latex (Reece et al., 2001), polystyrene (Thiele et al., 2003), and PLGA (Lutsiak et al., 2002; Newman et al., 2002). Maturation of DCs is precipitated by infection with pathogens or "danger" signals, such as necrotic tissues and their byproducts, and result in the presentation of the previously internalized and processed antigens in the context of MHC molecules for activation of naïve T lymphocytes (Matzinger, 1994; Banchereau and Steinman, 1998; Gallucci et al., 1999). These functional changes associated with DC maturation are correlated to the phenotypical changes including the downregulation of receptors involved in antigen internalization and adhesion as well as increased expression of MHC and co-stimulatory molecules such as CD40, CD80, and CD86 (Winzler et al., 1997; Banchereau and Steinman, 1998; Banchereau et al., 2000). An understanding of the mechanism of the biomaterial adjuvant effect will lead to selection of or design criteria for biomaterials for applications in tissue engineering or non-viral vaccine delivery by considering the effect of the biomaterial on associated immune responses. Specifically, biomaterials will be selected or designed to not support DC maturation where immune responses are undesirable, as for tissue engineered devices, while selected

or designed to support DC maturation where immune responses are desired, as for non-viral delivery vehicles for vaccines.

In this study, the effect of PLGA contact on the maturation of human peripheral blood monocyte-derived DC was examined as one possible explanation for the PLGA adjuvant effect previously observed (Matzelle and Babensee, 2004). The extent of maturation was assessed by changes in cell morphology, surface marker expression, allostimulatory capacity, and the ability of DCs to elicit delayed type hypersensitivity reaction as compared to appropriate controls. Results indicate that PLGA is a stimulus of DC maturation and that the form in which the biomaterial is presented to the DCs (MP vs film) influences the extent of maturation induced.

METHODS:

Poly(lactic-co-glycolic acid) microparticle and film preparation

Poly(lactic-*co*-glycolic acid) microparticles (MPs) were prepared by a single emulsion solvent evaporation technique adapted from a previously described method (Wake et al., 1998). Briefly, 500 mg of PLGA (molar ratio: 75:25, inherent viscosity: 0.69 dL/g in trichloromethane) (Birmingham Polymers, Birmingham, AL) was dissolved in 20 ml dichloromethane (DCM) (Sigma, St. Louis, MO) overnight at room temperature. On the second day, the PLGA-DCM solution was added to 200 ml 0.3% v/v aqueous poly(vinyl alcohol) (PVA) (Sigma), and homogenized for 2 min at 9000 rpm. Immediately after homogenization, 200 ml 2% (v/v) aqueous isopropanol (Sigma) was added to initiate precipitation, and the mixture stirred overnight at room temperature to evaporate the solvent. The resulting MPs were collected by centrifugation at 1000 rpm

for 10 minutes, washed twice in 2% isopropanol, and three times in distilled deionized H_2O (ddH_2O). The MPs were resuspended in ddH_2O and their size distribution characterized using a Coulter Multisizer II (Coulter Corporation, Miami, FL). Prior to use, MP suspension was UV sterilized for 1 hr, and stored at 4°C for no longer than 3 days.

Poly(lactic-*co*-glycolic acid) films were fabricated using a previously described casting technique without a porogen (Ishaug et al., 1997). Briefly, 10% w/v 75:25 PLGA was dissolved in DCM overnight at room temperature. The PLGA-DCM solution was poured onto a 50 mm Teflon Petri dish (Cole-Parmer, Vernon Hills, Illinois). Upon evaporation of the solvent and drying, the film was punched into appropriate size, and washed for 1 hr in ddH_2O, changing water every 15 min. The film was dried 30 min per side in a sterile laminar flow hood, and UV sterilized for 30 min per side prior to use. For culturing with cells, the film was secured to the bottom of the tissue culture plate with a segment of sterile silicone tubing (Cole-Parmer). The effective endotoxin content of a 4 mm PLGA film was determined to be 0.35 ± 0.12 EU/mL (Tachiyama et al., 1986). Previous study has shown that a minimum E. coli endotoxin concentration of 100 EU/mL was required for DC maturation (Jotwani et al., 2003).

Dendritic cell culture

Human blood was obtained from volunteers with informed consent, according to a protocol approved by the Institutional Review Board of Georgia Institute of Technology, #00A018. Dendritic cells were derived from human peripheral blood mononuclear cells (PBMCs) using a previously described method with some modifications (Romani et al.,

1996). Peripheral blood mononuclear cells were isolated from heparinized whole blood (333 U heparin/ ml blood) by differential centrifugation using Histopaque (Sigma). Cell layer containing mononuclear cells was collected and washed in phosphate buffer saline (PBS), and erythrocytes lysed with ammonium chloride (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), and washed again twice with PBS. Resulting PBMCs were resuspended at a concentration of $5x10^6$ cells/ ml in RPMI-1640 containing 25 mM HEPES and L-glutamine (Gibco BRL, Grand Island, NY) with 100 U/ ml penicillin-streptomycin (Cellgro, Herndon, VA) and 1% (v/v) heat inactivated filter sterilized autologous plasma (DC media). Cells were plated at in a volume of 10 ml/ plate in a 100x20 mm tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and incubated for 2 hrs in at 37˚C with 95% relative humidity and 5% CO_2 to select for adherent monocytes. After the incubation, plates were washed 3 times with warm DC media to remove non-adherent cells. The adherent cells were supplied with 10 ml DC media and incubated overnight without any cytokines. On day 1 of culture, media was replaced with fresh DC media containing 1000 U/ ml GM-CSF and 800 U/ ml IL-4 (R&D Systems, Minneapolis, MN). These cells were cultured for 4 days with one third of the media exchanged on day 3 of culture. On day 5 of culture, loosely- and non-adherent cells containing DCs were collected by centrifugation for 10 min at 1100 rpm, resuspended in DC media with 1000 U/ ml GM-CSF and 800 U/ ml IL-4, and plated in a 6 well plate (Corning, Corning, NY) at a concentration of $5x10^5$ cells/ well in 3 ml with or without stimulants. For the induction of mature DCs (mDCs), 1 μg/ ml lipopolysaccharide (LPS) (E. Coli 055:B5) (Sigma) was added, and cells with no additional maturation stimulants were used as iDCs. The effect of treatment of iDCs with PLGA was tested by adding

PLGA MPs at various MP: cell ratios (0.01:1, 0.1:1, 1:1) or plating cells on to a PLGA film previously positioned at the bottom of the plate well using a segment of silicone tubing. Two days later, on day 7 of culture, the cells were collected for further analysis.

Cell morphology

Morphology was monitored throughout the duration of culture by phase contrast microscopy of cells in culture or light microscopy of Cytospin preparations (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburgh, PA) stained with Differential Hematology Stain (Astral Diagnostics, West Deptford, NJ).

Cell surface marker expression

The levels of surface marker expression were monitored throughout the culture using flow cytometry (Ding et al., 1999). Cells were harvested, and resuspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM glucose, 30 mM HEPES) containing 1% (v/v) human serum albumin (HSA) and 1.5 mM $CaCl_2$, and stained with mouse anti- human monoclonal antibody against CD14 (clone UCHM1; IgG2aκ), CD40 (clone B-B20; IgG1κ), CD80 (clone BB1; IgMκ), CD86 (clone BU63; IgG1κ) (Southern Biotech, Birmingham, AL), CD83 (clone HB15a; IgG2b) (IO Test Immunotech Beckman Coulter, Marseille, France) HLA-DQ (clone TU169; IgG2aκ), or HLA-DR (clone TU36; IgG2aκ) (Becton Dickinson Pharmingen, San Diego, CA) for 1 hr at 4°C, and analyzed using BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using WinMDI 2.8 (Scripps Research Institute, La

Jolla, CA) and BD FACS DiVa Option 2.0 (Becton Dickinson Biosciences, San Jose, CA).

Transwell assay

The requirement for direct cell contact with PLGA MPs for DC maturation as measured by CD86 expression was determined using a transwell apparatus (24 mm diameter, 0.4 μm pore size, polyester membrane, Corning) based on a previously published protocol (Tsuji et al., 2000). Immature DCs on day 5 of culture were resuspended in DC media containing 1000 U/ ml GM-CSF and 800 U/ ml IL-4, and plated in the lower well of the apparatus with or without various maturation stimulants. On the upper well, media with or without cells or maturation stimulants were added. Upon 2 days of co-culture, cells on the lower wells were assessed for CD86 expression by flow cytometry as an indicator of DC phenotype.

TNFα blocking assay

In addition to transwell assays, *in vitro* blocking experiment was performed to evaluate the role of soluble factors released, namely TNFα, on DC maturation in response to treatment with PLGA. Immature DCs on day 5 were resuspended at a cell density of $1x10^6$ cells/ ml, and plated at 1 ml/ well in 24 well plates (Corning). These cells were either left untreated or treated with 1 μg/ ml LPS or 5:1 MP: cell ratio of PLGA MPs for 2 hrs at 37°C, and then 20 μg/ml isotype ($IgG_1κ$ isotype, clone 107.3, no sodium azide, low endotoxin, Becton Dickinson Pharmingen) or 20 μg/ml TNFα blocking (clone 1825.12, R&D Systems, Minneapolis, MN) antibodies added for

additional 22 hrs. Cells were collected by centrifugation and assessed for CD83 and CD86 expression, while the cell culture supernatant was collected, cleared and stored at -20°C until analysis by ELISA for TNFα content per manufacturer's directions (R&D Systems, Minneapolis, MN).

Mixed lymphocyte reaction (MLR)

Allostimulatory capacity of DCs to stimulate T cell proliferation in response to mismatches in MHC molecules was assessed using an allogeneic MLR, as previously described (Coligan et al., 1994). On day 7 of DC culture, allogeneic T cells were isolated from PBMCs by negative selection using Pan T cell magnetic isolation (Miltenyi Biotech, Auburn, CA) according to manufacturer's protocols, and purity checked by flow cytometry using anti-CD3 monoclonal antibody (clone SK7; IgG1, Becton Dickinson Pharmingen) staining. These cells were used as responder cells. T cells were resuspended in RPMI-1640 with 25 mM HEPES and L-glutamine (Gibco BRL) with 100 U/ ml penicillin-streptomycin (Cellgro) and heat inactivated filter sterilized 10% (v/v) human AB serum (Biowhittaker, Walkersville, MD) (complete RPMI-10 media) and plated at a concentration of $1x10^5$ cells/ well in a 96-well flat-bottomed plate (Corning). Dendritic cells cultured in GM-CSF and IL-4 with or without stimulants were resuspended at $1.6x10^5$ cells/ ml, and treated with 25 μg/ ml mitomycin C (Sigma) for 30 minutes to prevent their proliferation. Upon extensive washing with complete RPMI-10 media, DCs were resuspended in complete RPMI-10 media and added to responder cells in triplicates at graded DC: T cell ratios. Cells were co-cultured for 4 days, with the addition of 10 μM 5-bromo-2-deoxyuridine (BrdU) for the last 24 hrs of culture.

Dendritic cell-induced T cell proliferation was measured using BrdU colorimetric cell proliferation ELISA (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions.

PLGA MP-mediated Delayed Type Hypersensitivity (DTH) Reaction

Delayed type hypersensitivity reaction protocol was adapted from a previously published method (Gallucci et al., 1999), and approved by Georgia Institute of Technology Institutional Animal Care and Use Committee #A02028. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. C57BL6 male mice (6-8 wks old) (Charles River Laboratories, Wilmington, MA) were primed on day 0 with 100 μl of PBS, 1:1 dilution of complete Freund's adjuvant (CFA) (Sigma) in PBS, or PLGA MPs ($1.69x10^8$/ ml) each with or without 100 μg ovalbumin (OVA) (Sigma), as model antigen (6 mice/ group). After 21 days, the animals received a booster with 100 μl of the same carrier vehicle containing 0 or 100 μg OVA. In the case of CFA, incomplete Freund's adjuvant (IFA) (Sigma) was used in place of CFA. One week later, on day 28, the mice were challenged with an intradermal injection with 10 μg OVA in the left hind footpad in 10 μl PBS. On day 30, thickness and width of each hind footpads (challenged and contralateral unchallenged) were measured with an electronic digital caliper (Control Company, Friendswood, TX). The degree of DTH response was calculated as percent swelling, determined as the thickness of the challenged footpad over divided by the thickness of the contralateral unchallenged footpad.

Statistical Analysis

Statistical analysis was performed using general linear model ANOVA with Minitab software (Version 13.20, Minitab Inc., State College, PA), and pairwise comparison to control group (iDC or isotype) as appropriate, was made, with *p*-values of less than or equal to 0.05 considered to be significant.

RESULTS:

Immature dendritic cells cultured with PLGA microparticles or film show phenotypes consistent with maturation

To assess the effect of PLGA contact on DC maturation, human PBMC-derived iDCs were treated with PLGA MPs or films. Culture of peripheral blood monocytes with GM-CSF and IL-4 led to detachment of cells and formation of cell clusters, hallmarks of DC development, as observed using phase contrast microscopy (data not shown). As shown in Figure 5-1, morphology of iDCs treated with PLGA MPs or film was similar to that of DCs matured by LPS, with the presence of dendritic processes. Further, addition of PLGA MPs to iDCs led to the association, possibly internalization, of the MPs with the cells.

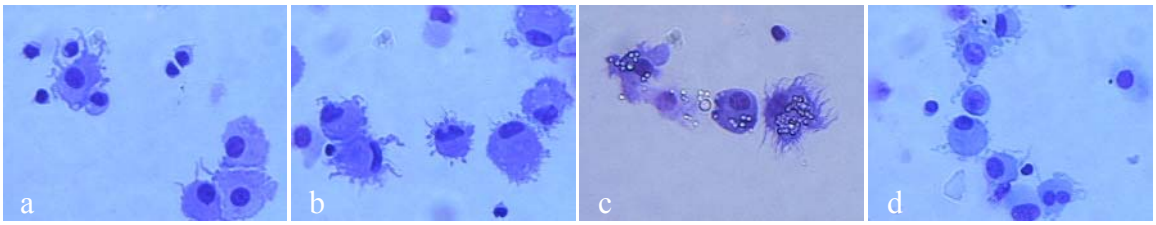

Figure 5-1: Dendritic cells treated with PLGA MPs or film possess cell morphology similar to DCs matured with LPS.
Immature DCs derived from peripheral blood monocytes in the presence of GM-CSF and IL-4 (a), cultured with PLGA MPs (c) or film (d) showed similar morphology to that of DCs matured by LPS (b), with the presence of dendritic processes. PLGA MPs used to treat DCs are associated with the cells, and possibly internalized. Original magnification: 40x.

Maturation of DCs is accompanied by an increase in expression of several cell surface markers including co-stimulatory molecules such as CD40, CD80, and CD86, and MHC class II molecules such as HLA-DQ and HLA-DR, and a marker of mDCs, CD83 (Zhou and Tedder, 1996; Banchereau and Steinman, 1998). To determine whether cell contact with PLGA MPs or film mediate phenotypical maturation of DCs, iDCs were treated with PLGA MPs or film for 2 days, and surface molecule expression was measured by flow cytometry and compared to the appropriate controls (Figure 5-2). As seen in Figure 5-2a, addition of LPS to iDCs resulted in a marked increase in the expression of co-stimulatory and MHC class II molecules, as well as in CD83, indicative of DC maturation. Upon exposure of iDCs to PLGA MPs or film, expression of CD40, CD80, CD86, CD83, HLA-DQ, and HLA-DR was increased compared to the control iDCs, in a dose-dependent manner (data not shown), but not to as great an extent as expressed by LPS-matured DCs. Further, cells treated with MPs expressed slightly higher levels of the maturation markers examined compared to cells treated with the film. Analysis of fold increase in marker expression due to biomaterial treatments indicated that CD83, CD86, and HLA-DQ were most significantly enhanced upon PLGA MP or film treatment of DCs (Figure 5-2b).

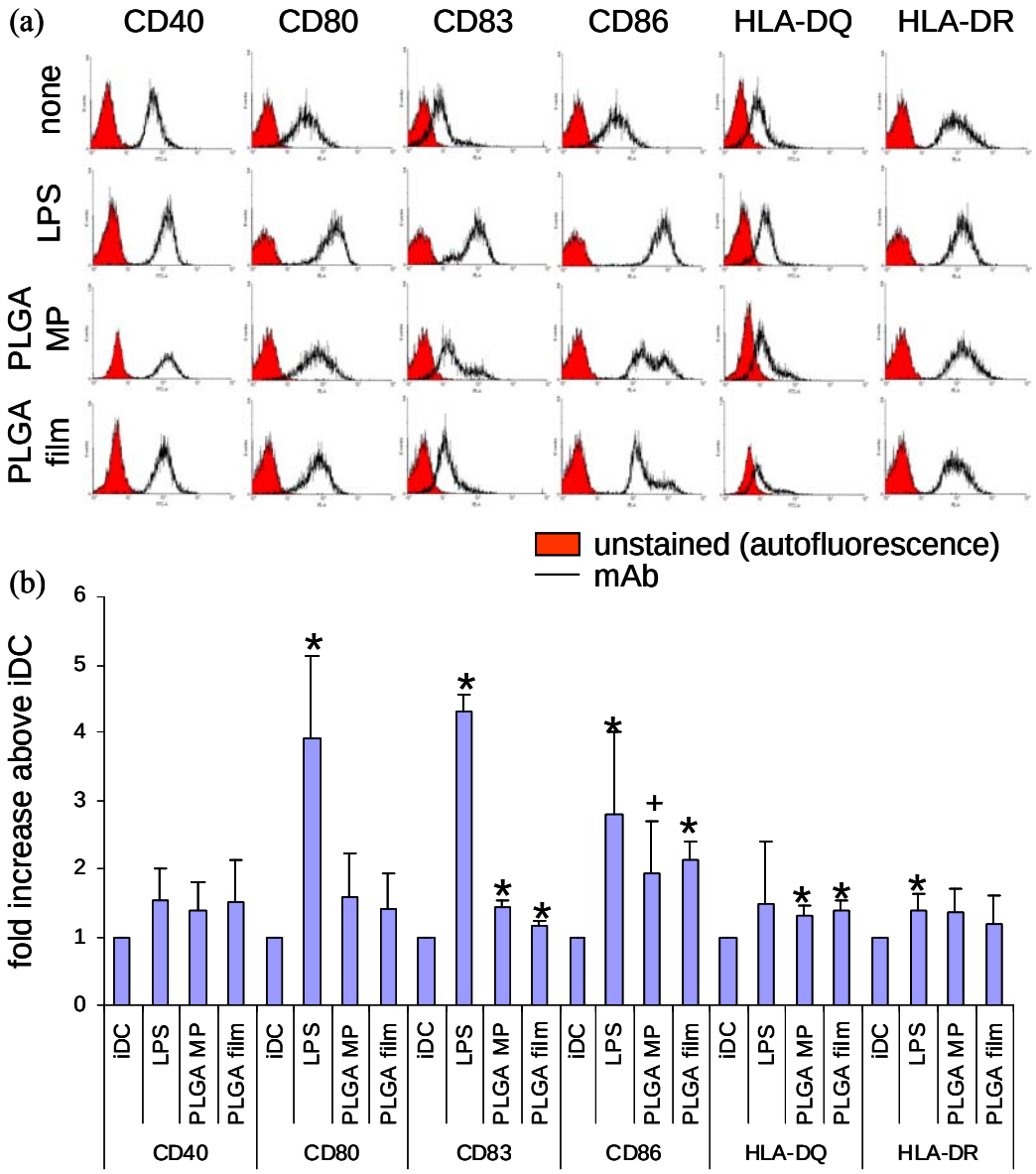

Figure 5-2: Dendritic cells treated with PLGA MPs or film moderately increase expression of co-stimulatory and MHC class II molecules.
Treatment of DCs with PLGA MPs or film resulted in shift of gMFI histograms indicating increased expression of maturation markers. This experiment was repeated three times with similar results, and representative histograms are shown (a). Fold increase in gMFI values for each treatment group as compared to that of iDC control revealed that treatment of DCs with PLGA MPs or film increase expression of CD83, CD86, and HLA-DQ at a statistically significant level (b). mean±SD, n=3, *: $p<0.05$. +: $p<0.1$ compared to iDC.

Upregulation of PLGA microparticle-induced CD86 expression by dendritic cells requires direct cell contact with biomaterial

To address whether the biomaterial-induced DC maturation requires direct contact between iDCs and PLGA MPs or whether soluble factors released by DCs in contact with PLGA MPs are sufficient to induce DC maturation, a transwell apparatus was used. As shown in Figures 5-3a, iDCs cultured with LPS regardless of direct or indirect contact exhibited higher level of CD86 expression as compared to iDCs, confirming solubility of LPS. Dendritic cells not in direct contact with PLGA MPs (samples iDC+PLGA MP/iDC, PLGAMP/iDC) expressed a similar level of CD86 as compared to iDCs without any maturation stimulants (samples media/iDC or iDC/iDC). In contrast, only iDCs cultured in direct contact with PLGA MPs (sample media/iDC+PLGA MP) showed upregulation in CD86 expression. Fold increase in expression of CD86 by differentially treated DCs validated the above conclusions at a statistically significant level (Figure 5-3b).

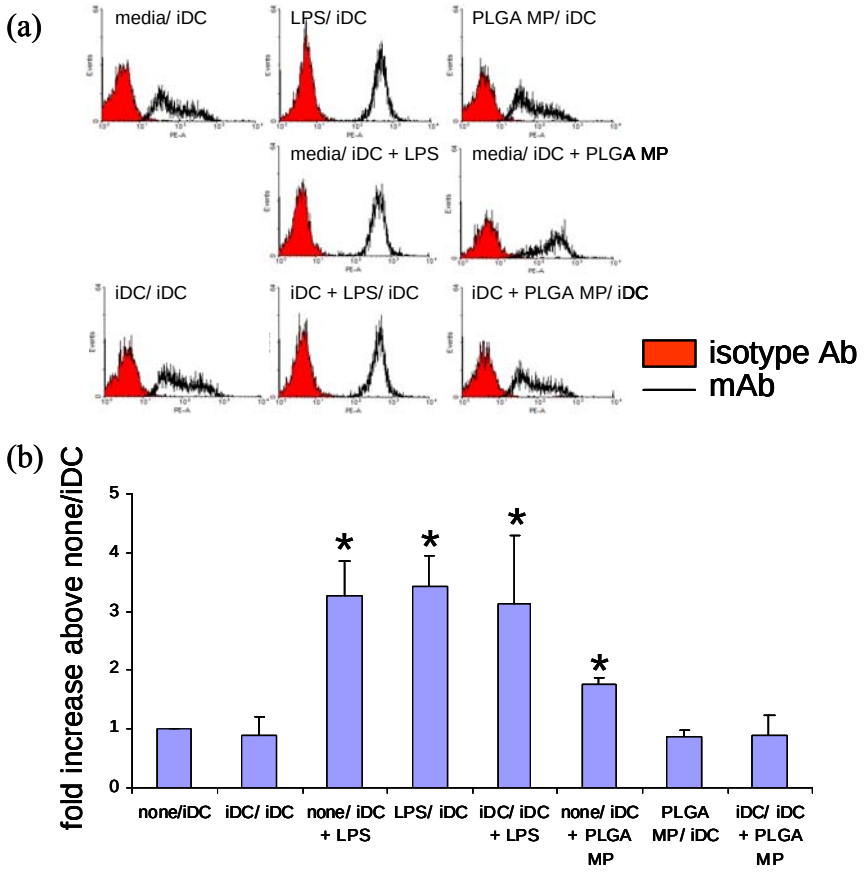

Figure 5-3: PLGA MP-induced DC maturation requires direct cell contact with PLGA MPs.

Only DCs in physical contact with PLGA MPs (media/iDC+PLGA MP) expressed higher levels of CD86, while DCs cultured with PLGA MPs separated by the transwell membrane (iDC+PLGA MP/iDC, PLGAMP/iDC) did not increase CD86 expression. The contents of the upper and lower wells of a transwell apparatus are indicated on the figures as "upper/ lower". This experiment was repeated three times with similar results, and representative results are shown (a). Fold increase in gMFI values for each treatment group as compared to that of none/iDC control confirmed findings from the histograms (b). mean±SD, n=3. *: $p<0.05$, compared to none/iDC.

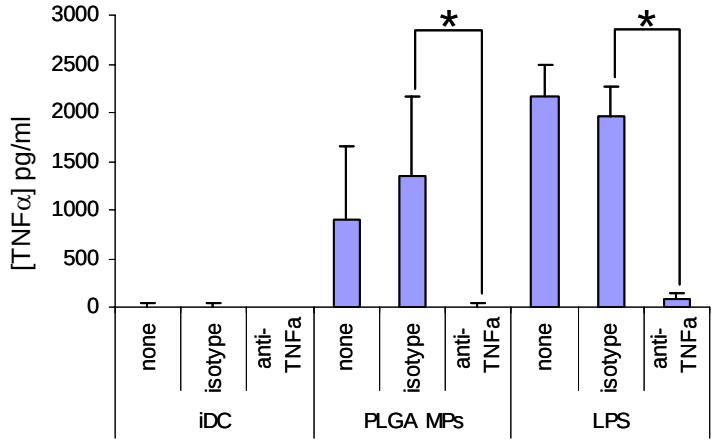

Figure 5-4: Increase in TNFα secretion induced by PLGA MPs or LPS is abrogated by the presence of anti-TNFα blocking antibody.
Dendritic cells treated with PLGA MPs or LPS increased secretion of TNFα as compared to iDCs. This increase was not affected by the addition of isotype control antibody but was abrogated in the presence of TNFα neutralizing antibody. mean±SD, n=3. *: $p<0.01$ compared to isotype control group, as indicated by the bracket.

While transwell assays indicated that DC maturation in response to PLGA MPs required direct contact between DCs and PLGA, blocking experiment was performed to confirm that PLGA MP-induced DC maturation was not mediated by autocrine effects of TNFα secreted by DCs cultured directly with PLGA MPs. As shown in Figure 5-4, DCs treated with LPS or PLGA MPs increased release of TNFα, which was not affected by the addition of isotype antibody. However, in the presence of TNFα blocking antibody, the amount of TNFα measured in the cell culture supernatant was significantly decreased. In addition, increase in CD83 and CD86 expression by DCs in response to PLGA MP-treatment was not reduced by the presence of TNFα neutralizing antibody (Figure 5-5). Pretreatment of iDCs with anti-TNFα antibody slightly decerased CD83 expression, while none of the other pretreatments resulted in alteration in the expression of CD83 or CD86 by iDCs or by PLGA MP-treated DCs.

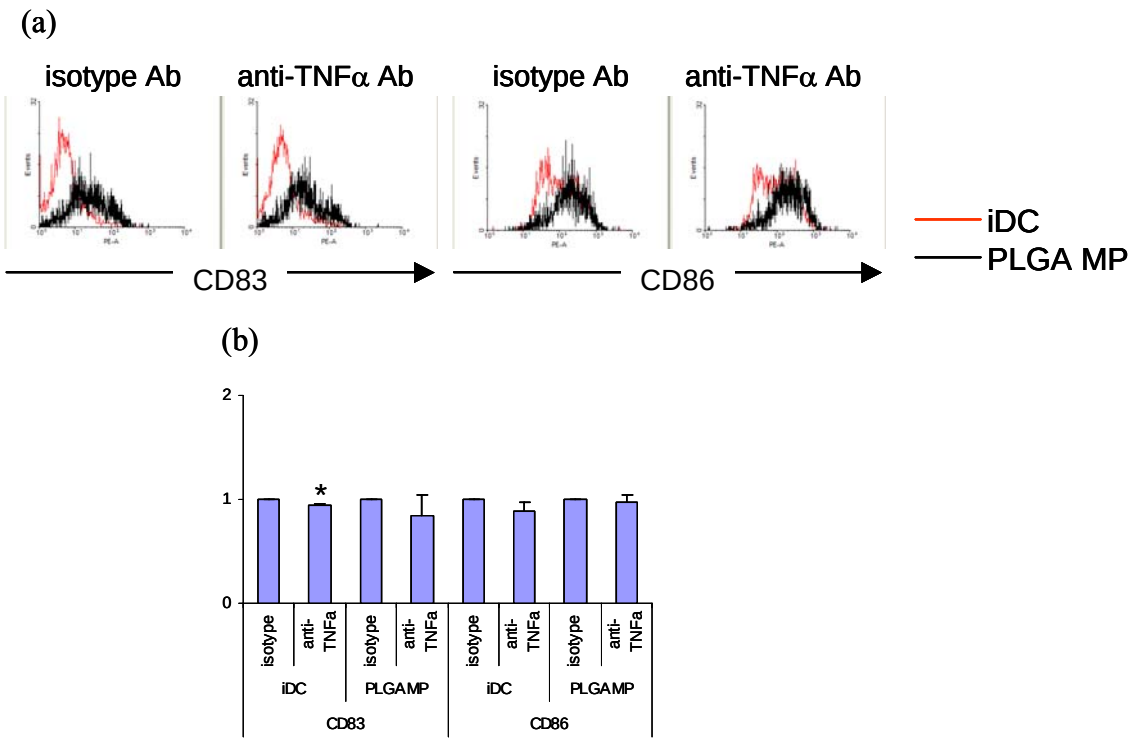

Figure 5-5: PLGA MP-induced DC maturation is not mediated by TNFα. Immature DCs left untreated or treated with PLGA MPs were incubated in the presence of isotype antibody or TNFα neutralizing antibody. Increase in CD83 and CD86 expression by PLGA MP-treated DCs was not reduced by the presence of TNFα blocking antibody. This experiment was repeated three times with similar results, and representative results are shown (a). Fold increase in gMFI values for anti-TNFα treated groups as compared to that of isotype antibody treated controls revealed that pretreatment of iDCs with the neutralizing antibody slightly decreased the expression of CD83 while none of the other treatments affected DC marker expression (b). mean±SD, n=3. *: $p<0.05$, compared to isotype control.

Dendritic cells cultured with PLGA microparticles are moderately allostimulatory in a mixed lymphocyte reaction

As mDCs are effective stimulators of T lymphocyte proliferation, the allostimulatory capacity of DCs treated with PLGA was measured by an allogeneic MLR. Immature DCs exposed to LPS (mDCs), PLGA MPs or film, or no additional stimulants (iDCs) were used as stimulators and allogeneic T lymphocytes were used as responders at various stimulator: responder ratios. As shown in Figure 5-6, LPS-matured DCs were highly allostimulatory in a stimulator: responder ratio-dependent manner, while control iDCs induced little or no T lymphocyte proliferation. Dendritic cells treated with PLGA MPs were capable of stimulating an MLR, but to a lesser extent than LPS-matured DCs. However, cells treated with PLGA film did not induce significant T cell proliferation over that induced by control iDCs.

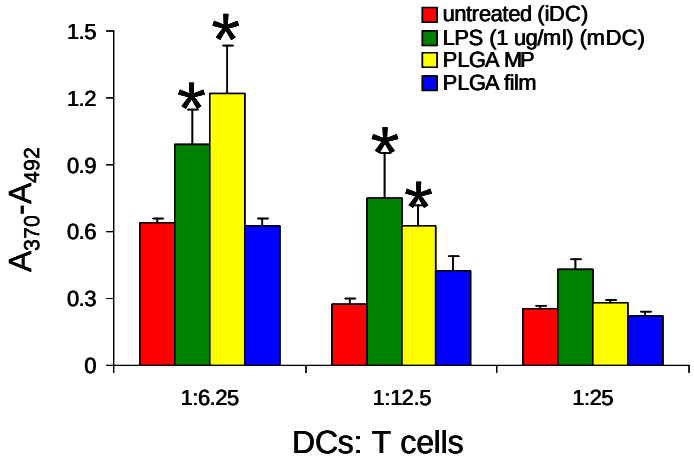

Figure 5-6: Dendritic cells cultured with PLGA MPs are moderately allostimulatory in an MLR.
Dendritic cells matured with LPS are highly allostimulatory, whereas iDCs are not. Dendritic cells treated with PLGA MPs elicited a moderate proliferation of allogeneic T cells, but DCs cultured on PLGA film were not significantly allostimulatory above that of iDCs. This experiment was repeated four times with similar results, and representative results are shown. mean±SD, n=4 wells. *: $p<0.05$ compared to iDC.

PLGA microparticles elicit a moderate delayed type hypersensitivity reaction in mice

As DCs exposed to PLGA MPs were capable of upregulating co-stimulatory and MHC class II molecules and stimulating allogeneic T lymphocyte proliferation *in vitro*, the ability of PLGA MPs to stimulate DCs to become antigen presenting cells *in vivo* to initiate a T cell-mediated response was assessed using a DTH reaction against a model antigen. Animals were primed with CFA, PBS, or PLGA MPs with or without OVA, and boosted 3 weeks later with the same carrier vehicle (except IFA in place of CFA). A week later, the animals were challenged with an intradermal injection in the footpad with OVA in PBS. The degree of swelling was measured 2 days after the challenge. Ovalbumin delivered with PLGA MPs induced a similar level of DTH as OVA delivered with the strong adjuvant, CFA (Figure 5-7).

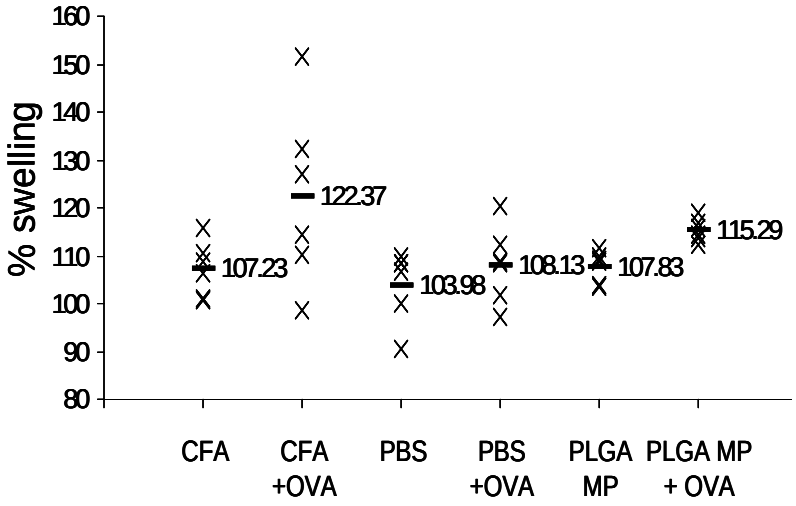

Figure 5-7: PLGA MPs elicit a moderate DTH reaction in mice.
Ovalbumin delivered with PLGA MPs induced a similar level of DTH as OVA delivered with the strong adjuvant, CFA. Results are reported as % swelling (thickness of challenged footpad (mm) / thickness of unchallenged contralateral footpad (mm) x 100%). Each symbol represents an animal, and the mean % swelling is indicated, and represented by a bar. Six mice were placed into each group.

DISCUSSION:

Immunorecognition of cellular components shed from a tissue engineered construct may result in failure of an implanted device. Previously, PLGA, a commonly used biomaterial in tissue engineered devices, was shown to act as an adjuvant to heighten the adaptive immune response towards associated antigens (Matzelle and Babensee, 2004). Dendritic cells function as a bridge between innate and adaptive immunity wherein adjuvants exploit the fact that the innate immune response prods the slower acting adaptive immune response due to its effects on the maturation of DCs such that they become efficient antigen presenting cells and T cell stimulators. Therefore, we focused on the extent of PLGA-induced maturation of DCs as a possible explanation for the observed adjuvant effect of PLGA (Matzelle and Babensee, 2004). In this study, we observed that 75:25 PLGA MPs or films enhanced the maturation of human monocyte-derived DCs as assessed by characteristic cell morphology, upregulation of cell surface marker expression, allostimulatory capacity, and DTH induction *in vivo*.

Exposure of iDCs to LPS led to expected, marked increase in cell surface marker expression as compared to untreated iDCs indicating DC maturation. Treating iDCs with PLGA MPs resulted in an increase in expression of cell surface markers to a level between that of untreated iDCs and LPS-matured DCs. Culturing iDCs on PLGA film resulted in an increase in surface marker expression but to a lesser extent than the increase observed for cells treated with PLGA MPs. In this study, iDCs cultured with PLGA MP most significantly increase CD83, CD86, and HLA-DQ. Since CD40 upregulates CD86 expression (Banchereau and Steinman, 1998), it is possible that PLGA MP-induced maturation of DCs may be regulated by CD40. Likewise, iDCs treated with

PLGA MPs were similar in their allostimulatory efficiency as compared to that of cells matured with LPS. In contrast, iDCs cultured on PLGA film did not elicit a high proliferation of T lymphocytes, suggestive of lower induction of maturation. These results imply that the form in which the biomaterial is presented to DCs (MPs vs film) may play a role in the extent of DC maturation, suggesting a role of particle internalization in DC maturation.

While it has been shown that PLGA MPs can be phagocytosed by DCs (Lutsiak et al., 2002; Newman et al., 2002), internalization of PLGA MPs by DCs was not ascertained in the study presented herein, although association of MPs with cells was observed. Since internalization of particles may enhance DC maturation (Sallusto et al., 1995; Singh and O'Hagan, 1999), PLGA presented in a MP form may be more capable of stimulating DC maturation compared to PLGA presented as a flat surface. In the DC culture system used in the study presented here, PLGA MPs were likely internalized by DCs, as the particles were small in size, with an average diameter of 4 μm. Further, phagocytosis of fluorescent microspheres of similar size was confirmed by confocal laser microscopy (data not shown). Cytospin preparations (Figure 5-1c) and phase contrast micrographs (data not shown) demonstrate the association of the PLGA MPs with the DCs. Given the phagocytosable size of the PLGA MPs used here and the transwell assay results indicating the direct biomaterial contact requirement for DC maturation, it is speculated that the phagocytosis of PLGA MPs contributed to the greater extent DC maturation as compared to treatment with PLGA film (exemplified especially in the MLR results). Additional studies are underway to evaluate the actual contribution of MP phagocytosis on DC maturation by inhibiting phagocytosis with pharmacological agents,

and also by comparing the degree of maturation of DCs upon culturing with MPs of phagocytosable or non-phagocytosable size with equal total biomaterial surface area exposed to the cells (CHAPTER7). However, the biomaterial effect on DC maturation does not appear to be due only to biomaterial phagocytosis since treatment with PLGA films induced phenotypical maturation of DCs and preliminary results indicate that there is a differential effect depending on the type of biomaterial used (Babensee and Paranjpe, 2005).

Results reported herein support other studies which have showed that PLGA MPs can be internalized by DCs, localize to the phagosomal vesicles (Walter et al., 2001), and lead to their maturation (Thiele et al., 2001). On the contrary, a recent report in the murine system examining the adjuvant effect of PLGA in mediating DC maturation in the presence of an antigen has shown that while PLGA particles were able stimulate an antigen-specific *in vivo* immune response, they were unable to activate DCs *in vitro* (Sun et al., 2003). The differences in the results from this study may be due to the model system used, presence or absence of an antigen, differences in *in vitro* methods for DC culture and co-culture with MPs, PLGA particle properties, and the ratio of particles to cells. The ability of PLGA MPs to mediate adjuvant effect *in vivo* was measured by the induction of DTH response, a T helper 1 (Th1) cell mediated immune response. The DTH results reported herein demonstrated that the delivery of OVA with PLGA MPs can elicit a similar level of DTH response as OVA delivered with CFA, a strong adjuvant. This implies that PLGA MPs contribute to the activation of T cells, and hence the development of DCs as efficient antigen presenting cells *in vivo* for the presentation of co-delivered OVA to prime T lymphocytes. While these results suggest that PLGA MPs

can support a Th1 response *in vivo*, the ability of the PLGA MPs to elicit a Th2 response was not examined in this study. Other studies investigating the nature of Th response induced by PLGA MPs have shown that OVA peptide encapsulated in PLGA MPs elicit a Th1 immune response whereas the co-delivery of soluble/adsorbed OVA with PLGA MPs result in a Th2 immune response (Matzelle and Babensee, 2004). The disparity between the two results may be due to the differences in the form of the antigen used (peptide vs protein), the mode of antigen co-delivery (encapsulated vs adsorbed), and the method of determining the nature of the Th response (serum IgG isotypes levels vs cytokines secreted by *in vivo* primed lymphocytes following *in vitro* second challenge with antigen). Controlling helper T cell polarization via DC phenotype as directed by biomaterials is an attractive approach to improve host tolerance to a tissue engineered device. Stimulated DCs release a cytokine profile for the polarization of T cells to Th1, Th2 or regulatory T cells which is dependent on the activation by a particular pathogen and other inflammatory tissue factors (Kalinski et al., 1999). Previous study has shown that DCs generated *in vitro* which secreted IL-10 (a regulatory and Th2 cytokine), but not IL-12 (a Th1 cytokine), prevented allograft rejection in a mouse model, suggesting that polarized cytokine production plays a role in tolerogenicity (Gao et al., 1999). Further, co-stimulatory molecules CD80 and CD86 may regulate Th1/ Th2 polarization (Coyle and Gutierrez-Ramos, 2001). The role of Th1/ Th2 polarization and the regulatory T cell cytokine profile in acceptance of transplants and induction of tolerance is incompletely understood. Nonetheless, controlling of Th polarization through the biomaterial effects on DC phenotype is an intriguing strategy for inducing acceptance of tissue engineered devices. The characterization of cytokine profile produced by lymphocytes in the MLR

supported by biomaterial-treated DCs will provide insight into whether biomaterials can differentially drive tolerance for the host acceptance of a tissue engineered construct.

The experiments presented herein support the premise that the PLGA adjuvant effect is (at least partially) explained by its ability to support DC maturation. The extent of DC maturation was dependent on the form in which the biomaterial was presented to iDCs; higher level for PLGA presented as phagocytosable MPs vs nonphagocytosable film, although treatment with the PLGA film did induce phenotypical maturation. In addition, PLGA MPs were capable of eliciting a modest DTH reaction, indicating *in vivo* maturation of DCs such that they become efficient antigen presenting cells. This study serves as an initial effort in understanding the mechanisms of the adjuvant effect associated with biomaterials used in tissue engineering and the role of DCs in mediating such an effect. The elucidation of cellular mechanisms involved in the immune response against a tissue engineered construct will provide insight into design criteria for biomaterials, as well as immunomodulatory strategies, for enhanced integration of the device into host living systems.

CHAPTER 6

MOLECULAR ASPECTS OF MICROPARTICLE PHAGOCYTOSIS BY DENDRITIC CELLS*

INTRODUCTION:

Dendritic cells (DCs) are professional antigen presenting cells (APCs) central in the control of the immune response by bridging the innate and adaptive immunity (Banchereau and Steinman, 1998; Banchereau et al., 2000). In the immature state, DCs reside in peripheral tissues where they monitor the surrounding microenvironment by capturing and internalizing antigens by pinocytosis as well as by receptor-mediated endocytosis (Banchereau and Steinman, 1998). Dendritic cells undergo a maturation process upon encountering a stimulus such as infection by pathogens or "danger" signals, including byproducts of necrosis and inflammation or tissue damage, and present the previously internalized and processed antigens in the context of MHC molecules for activation of naïve T lymphocytes (Matzinger, 1994; Banchereau and Steinman, 1998; Gallucci et al., 1999). In addition to particulates that DCs may encounter endogenously, synthetic materials including particles of latex (Matsuno et al., 1996; Reece et al., 2001), polystyrene (Thiele et al., 2003), and poly(lactic-*co*-glycolic acid) (PLGA) (Lutsiak et al., 2002; Newman et al., 2002) have been shown to be phagocytosed by DCs. The maturation of DCs result in functional and associated phenotypical changes, such as the decrease in the expression of endocytic and adhesion receptors, increase in the expression of MHC and co-stimulatory molecules (Winzler et al., 1997; Banchereau and Steinman,

* Modified from Yoshida M, Babensee JE. Molecular aspects of microparticle phagocytosis by dendritic cells. Manuscript to be submitted to Journal of Biomaterials Science. Polymer edition.

1998; Banchereau et al., 2000), and loss of phagocytic activity (Sallusto and Lanzavecchia, 1994).

The use of polymeric microspheres and nanospheres for vaccines and controlled drug delivery systems have prompted the understanding of the role of DCs and their phagocytic capacity in mediating the immune response against co-delivered antigens (Matsuno et al., 1996; Reece et al., 2001; Lutsiak et al., 2002; Newman et al., 2002; Prior et al., 2002). Uptake of polymeric particles is dependent on material properties including size, hydrophobicity, and surface charge (Tabata and Ikada, 1991). While biomaterial particle internalization by DCs has been observed, the details of uptake mechanism remain unclear, although PLGA nanosphere uptake by human DCs has been shown to be inhibited by pretreating the cells with cytochalasin B (Lutsiak et al., 2002). Other studies have demonstrated that endocytosed PLGA microspheres localize to the endosomal compartments and the cytoplasm (Audran et al., 2003), and endocytosed latex nanospheres either remain as singular beads near the surface, or as small aggregates, co-localized with MHC class II molecules (Reece et al., 2001). Moreover, recent reports have focused on the role of adsorbed proteins and opsonins on biomaterial particle surface on DC phagocytosis (Thiele et al., 2003). While uptake of polymer particles coated with adsorbed proteins may occur via receptors, reports of receptor-mediated uptake to date has been focused on microparticles (MPs) of more biological origin such as starch, mannan (Artursson et al., 1998), and zymosan (Ezekowitz et al., 1985).

We have previously shown that PLGA exerts an adjuvant effect in the context of tissue engineering, as measured by the increase in the specific humoral response against a co-delivered model antigen (Matzelle and Babensee, 2004) which was dependent on the

physical form of PLGA (Bennewitz and Babensee, 2005). As a possible explanation of this observed adjuvanticity, the effect of PLGA contact on the maturation of human peripheral blood monocyte-derived DCs was investigated. The effects of films as well as MPs of PLGA were studied, with MPs serving as a model of degradation product generated from implanted tissue engineered scaffolds, in addition to being a common vaccine and controlled drug delivery system. Culturing of human DCs with PLGA MPs resulted in increased allostimulatory capacity and surface expression of co-stimulatory and MHC molecules, which was contact-dependent (Yoshida and Babensee, 2004). As these results suggest that PLGA MPs are stimuli of DC maturation and that the MPs were internalized, we sought to further investigate the molecular basis for polymer MP uptake by human DCs and to determine to what extent particle phagocytosis played a role in DC maturation. To visualize the internalization of these microspheres by DCs, model fluorospheres of polystyrene, available commercially, or coumarin 6-loaded PLGA MPs were used for validations via flow cytometry and assessment of molecular aspects of this phagocytosis. Maturation of DCs upon culturing with polystyrene or PLGA MPs was further evaluated by quantifying the release of the autocrine maturation cytokine, TNFα, by DCs in response to varying doses of particles used to treat the DCs.

METHODS:

Poly(lactic-co-glycolic acid) microparticle preparation

Preparation of PLGA MPs has been described in detail elsewhere (Yoshida and Babensee, 2004). Briefly, PLGA (molar ratio: 75:25, inherent viscosity: 0.69 dL/g in trichloromethane) (Birmingham Polymers, Birmingham, AL) dissolved in dichloromethane (DCM) (Sigma, St. Louis, MO) was added to 0.3% v/v aqueous poly(vinyl alcohol) (PVA) (Sigma), and homogenized. For fluorescently labeled PLGA MPs, coumarin 6 (Sigma) dissolved in DCM was added to the homogenization mixture. Precipitation was initiated with isopropanol (Sigma) and the solvent was evaporated overnight under constant stirring. Resulting MPs were collected by centrifugation and washed extensively with isopropanol followed by double distilled water. Size distribution of MPs was determined using Coulter Multisizer II (Coulter Corporation, Miami, FL) with a peak diameter of approximately 3 μm. Prior to use, MPs were sterilized by UV radiation for 1 hr, and stored at 4°C for no longer than 3 days.

Dendritic cell culture

Dendritic cells were derived from human peripheral blood mononuclear cells (PBMCs) as previously described (Yoshida and Babensee, 2004). Human blood was obtained from volunteers with informed consent, according to a protocol approved by the Institutional Review Board of Georgia Institute of Technology, #00A018. Briefly, PBMCs were isolated from heparinized whole blood by centrifugation using Histopaque (Sigma). Cell layer containing mononuclear cells was washed in phosphate buffer saline (PBS), erythrocytes lysed, and washed again twice with PBS. Resulting PBMCs were

resuspended at a concentration of $5x10^6$/ ml in RPMI-1640 containing 25 mM HEPES and L-glutamine (Gibco BRL, Grand Island, NY) with 100 U/ ml penicillin-streptomycin (Cellgro, Herndon, VA) and 10% heat inactivated filter sterilized fetal bovine serum (FBS) (Gibco BRL) (DC media). Monocytes were selected by plastic adherence for 2 hrs at 37˚C with 95% relative humidity and 5% CO_2. Upon washing the plates with warm DC media to remove non-adherent cells, the remaining adherent monocytes were resuspended in 10 ml DC media with 2000 U/ ml GM-CSF and 1600 U/ ml IL-4 (Peprotech, Rocky Hill, NJ) and left undisturbed in an incubator at 37˚C with 95% relative humidity and 5% CO_2 until day 5 of culture. On day 5, loosely- and non-adherent cells containing iDCs were collected, resuspended in DC media with 1000 U/ ml GM-CSF and 800 U/ ml IL-4, and used as iDCs in the various experiments described below.

Blocking of dendritic cell fluorosphere uptake

To visualize the uptake of microspheres by DCs, iDCs collected on day 5 were resuspended at a concentration of $5x10^5$/ ml and plated into a 6 well plate. Prior to the addition of fluorospheres, iDCs were treated with or without varying concentrations of EDTA (Gibco BRL), varying concentrations of trypsin (Spectrum Chemical, New Brunswick, NJ), 20 μg/ ml cytochalasin D (Sigma), or 5 μl/ ml DMSO (Sigma) for 1 hr at 37°C. Alternatively, DCs were incubated for 1 hr at 4°C. Upon pretreatment, DCs were treated with 1:1 MP: cell ratio of 3 μm polystyrene fluorospheres (Fluoresbrite Plain YG microspheres, Polysciences, Warrington, PA) (FYG microspheres) or coumarin 6-loaded PLGA MPs (cPLGA MPs), and allowed to incubate for 4 hrs at 37°C in the presence of

the pretreatment agents or at 4°C. After the incubation of the cells with fluorospheres, cells were collected by centrifugation, washed 3 times with ice cold Hank's HEPES buffer with 1% human serum albumin (Calbiochem, San Diego, CA) to remove extracellularly associated fluorospheres, and assessed for fluorosphere uptake by flow cytometry or confocal laser scanning microscopy, as detailed below.

Flow cytometry assessment of fluorosphere uptake

Upon washing the DCs to dissociate any non-internalized fluorospheres associated on the cell surface, DCs were resuspended in ice cold Hank's HEPES buffer with 1% human serum albumin and analyzed by BD Vantage flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA). Dendritic cells were gated based on forward and side scatters and those with high fluorescence (FL) in FITC channel were considered as DCs with associated fluorospheres.

Confocal laser scanning microscopy of fluorosphere internalization by dendritic cells

To visually confirm the DC internalization of microspheres, confocal laser scanning microscopy was performed. Immature DCs on day 5 were washed and placed on cover slips previously coated by a total of 15 min incubation in 0.01% poly-L-lysine solution (Sigma), and allowed to adhere to the cover slips for 24 hrs at 37°C. On the following day, the non-adherent cells were removed, and the adherent cells were fixed using 2% paraformaldehyde (Sigma), then permeabilized with 1% Triton X-100 (Sigma). Upon washing, DCs were stained for actin filaments using rhodamine labeled phalloidin (Molecular Probes, Eugene, OR), counterstained for the nucleus using Hoechst 33342

(Molecular Probes), and visualized using Zeiss LSM 510 Confocal Microscope (Zeiss). Z-stack images were also acquired to record changes throughout the cell.

TNFα release by dendritic cells cultured with microspheres

On day 5 of culture, iDCs were collected and resuspended at a concentration of $1x10^6$/ ml, and plated at a density of 1 ml/ well into a 24 well plate. Dendritic cells without any additional stimulants were used as negative controls to yield iDCs, and those treated with 1 μg/ ml lipopolysaccharide (LPS) (E. Coli 055:B5) (Sigma) were used as positive controls to yield mature DCs (mDCs). The maturation effect of treatment with FYG microspheres or PLGA MPs was evaluated by adding the microspheres at varying concentrations to iDCs. Upon 24 hrs of culturing DCs with the fluorospheres, the cell culture media was collected, cleared of cells and MPs, and stored at -20°C until analysis. The amount of TNFα in the media was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

Statistical Analysis

Statistical analysis was performed using general linear model ANOVA with Minitab software (Version 13.20, Minitab Inc., State College, PA), and pairwise comparison to appropriate control group as indicated was made, with *p*-values of less than or equal to 0.05 considered to be significant.

RESULTS:

Three micron fluorospheres are internalized by immature dendritic cells

Confocal laser scanning microscopy was used to visualize the internalization of FYG microspheres or cPLGA MPs of approximately 3 μm in diameter by iDCs. Upon incubation with the fluorescent particles for 4 hrs, DCs had internalized at least one, if not several fluorospheres, as seen by the surrounding of the fluorospheres by the actin filaments. Orthogonal images were captured to confirm that the fluorospheres were inside the cells, rather than externally attached to the cell surface (Figure 6-1).

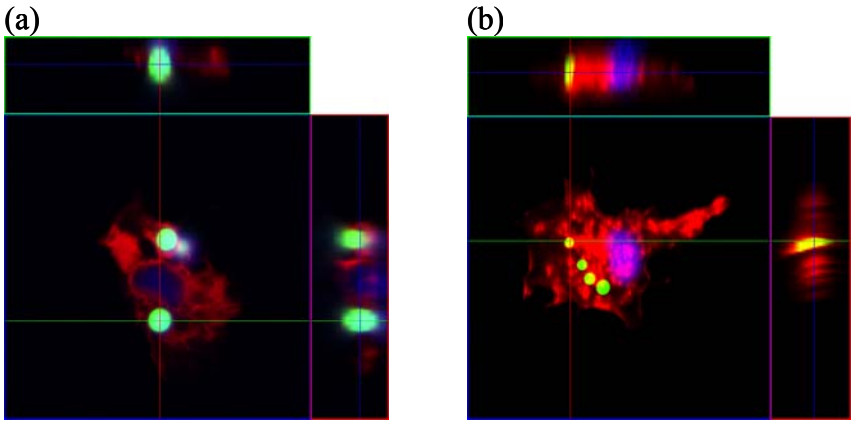

Figure 6-1: Dendritic cells internalize FYG microspheres (a) or cPLGA MPs (b). Dendritic cells cultured with FYG microspheres (a) or cPLGA MPs (b) for 4 hrs at 37°C were washed and loaded onto poly-L-lysine coated cover slips. Fixed DCs were permeabilized, and stained for actin filaments and nucleus using with rhodamine phalloidin and Hoechst 33342, respectively, and analyzed by confocal laser scanning microscopy. Original magnification: (a) 63x, (b) 40x. This experiment was repeated three times with similar results, and representative results are shown.

To further evaluate the uptake of fluorescent microspheres, the DCs were pretreated either with cytochalasin D or incubated at 4°C for 1 hr prior to the addition of the fluorospheres. The uptake of the fluorospheres by DCs as measured by flow cytometry was lowered by the pretreatment of the cells with cytochalasin D or incubation with fluorospheres at 4°C (Figure 6-2). Viability was not greatly affected by the pretreatment of cells with cytochalasin D (98.91% viability) or incubation at 4°C (98.75 % viability) as compared to cells left untreated at 37°C (98.05% viability). As shown in Figure 6-2a-c, DCs cultured without any fluorescent particles exhibit a sharp, tall peak at a very low FL value, which corresponds to their autofluorescence, without any additional peaks at higher FL values. Dendritic cells cultured with FYG microspheres show distinct sharp peaks at higher FL values whereas those cultured with cPLGA MPs show one broader peak spanning a wider spectrum of FL values. Dendritic cells cultured at 37°C without any chemical pretreatment show highest fraction of DCs positive for fluorospheres (Figure 6-2d). Dendritic cells incubated with cPLGA MPs or FYG microspheres at 37°C without any chemical pretreatment showed mean fluorescent intensities (MFI) of 590.72 and 333.84, respectively. For DCs cultured with FYG microspheres, pretreatment of cells at 4°C (MFI: 260.49) or with 20 μg/ ml cytochalasin D (MFI: 260.27) statistically significantly decreased the percentage of DCs that were positive for fluorescent microspheres. For DCs cultured with cPLGA MPs, only the pretreatment of cells with cytochalasin D (MFI: 270.35), but not incubation at 4°C (MFI: 574.59), significantly decreased the uptake of the MPs by DCs. Pretreatment of DCs with DMSO, the vehicle used for cytochalasin D, did not affect particle uptake or cell viability (data not shown).

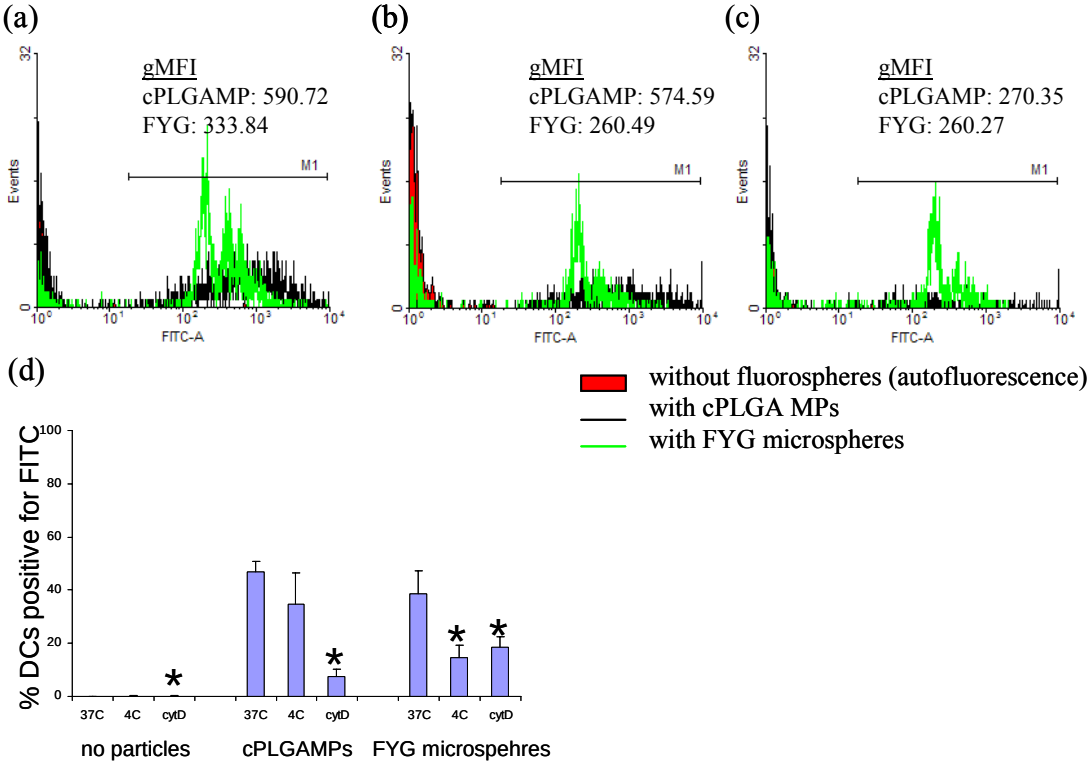

Figure 6-2: Pretreatment of DCs with cytochalasin D or incubation at 4°C decreases fluorosphere uptake.

On day 5 of culture, iDCs were left untreated at 37°C (a), incubated at 4°C (b), or pretreated with 20 μg/ ml cytochalasin D at 37°C (c) for 1 hr, and cultured with 1:1 MP:cell ratio of fluorospheres for 4 hrs. Fluorescence associated with DCs was evaluated by flow cytometry. This experiment was repeated three times with similar results, and representative results are shown.

Figure (d) represents the average of percentage of DCs positive for fluorescence (from fluorospheres). mean±SD, n=3. *: $p<0.05$ as compared to cells without pretreatment agents, incubated at 37°C.

Pretreatment of immature dendritic cells with EDTA or trypsin decrease fluorosphere uptake

To begin to address the mechanism of fluorosphere uptake by iDCs, iDCs were pretreated with varying concentrations of EDTA or trypsin prior to incubation with the fluorospheres. Pretreatment of iDCs with EDTA (Figure 6-3a) or trypsin (Figure 6-3b) resulted in decreased uptake of both FYG microspheres and cPLGA MPs in an EDTA or trypsin dose-dependent manner. At higher concentrations, EDTA and trypsin significantly decreased the uptake of both FYG microspheres and cPLGA MPs by DCs.

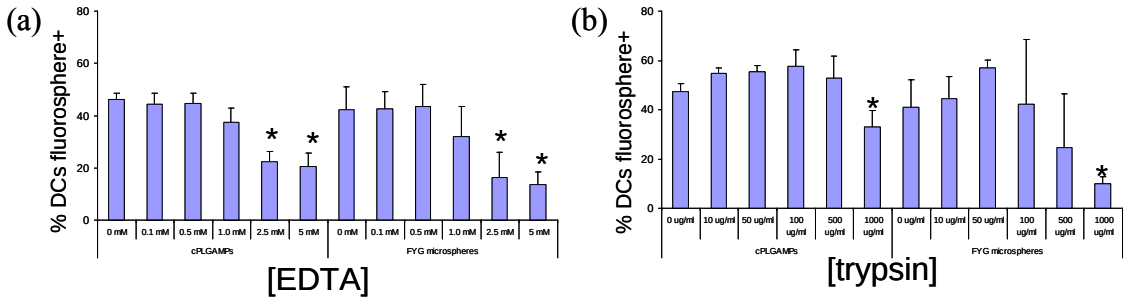

Figure 6-3: Internalization of fluorospheres by DCs is dependent on extracellular calcium concentration and sensitive to trypsin.
On day 5 of culture, iDCs were pretreated with indicated concentrations of EDTA (a) or trypsin (b) for 1 hr at 37°C. Fluorospheres were added at a fluorosphere: cell ratio of 1:1 for 4 hrs at 37°C. DCs were washed 3 times in ice cold buffer, and then assessed for associated fluorosphere fluorescence by flow cytometry. mean±SD, n=3. *: $p<0.05$ compared to 0 mM EDTA or 0 μg/ml trypsin.

Dendritic cells treated with FYG microspheres or PLGA microparticles secrete TNFα in a dose-dependent manner

As we have previously observed that exposure of iDCs to PLGA MPs result in their maturation as assessed by upregulation of co-stimulatory and MHC class II molecules and enhanced allostimulatory capacity (Yoshida and Babensee, 2004), secretion TNFα, an autocrine factor for DC maturation, was measured as another indicator of DC maturation upon treatment with PLGA MPs. Dendritic cells cultured in the presence of varying MP:cell ratio of FYG microspheres or PLGA MPs secreted amounts of TNFα in between the levels secreted by iDCs and mDCs, in a microsphere dose-dependent manner (Figure 6-4). In comparison to DCs cultured with FYG microspheres, DCs cultured with PLGA MPs secreted higher amounts of TNFα. At the highest MP:cell ratio of 10:1, amount of TNFα secreted by DCs exposed to PLGA MPs was significantly higher than that secreted by iDCs cultured alone, supporting our previous studies in which PLGA resulted in a maturation effect on DCs (Yoshida and Babensee, 2004).

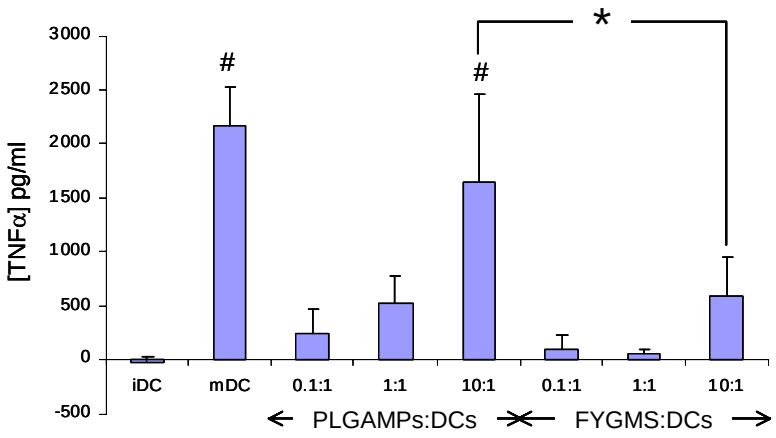

Figure 6-4: Treatment of DCs with FYG microspheres or PLGA MPs induces TNFα secretion in a microsphere dose-dependent manner.
On day 5 of culture, iDCs were left untreated or treated with indicated doses of FYG microspheres or PLGA MPs, or with 1 μg/ ml LPS as a positive control. After 24 hr incubation, cell culture media was cleared and stored at -20°C until analysis. mean±SD, n=3. #: $p<0.05$ compared to iDCs, *: $p<0.05$ between FYG microsphere and PLGA MP-treated groups at microsphere: cell ratio at 10:1.

DISCUSSION:

The capture and internalization of antigens by DCs lead to processing and presentation of associated peptides to the T lymphocytes for the initiation of the adaptive immune response (Banchereau et al., 2000). Because of this ability of DCs to initiate immune response and their ability for phagocytosis, antigen delivery using polymeric particles targeted for DC uptake has gained much focus as a strategic approach to vaccination (Lutsiak et al., 2002; Audran et al., 2003). Conversely, these characteristics of DCs and similarly, macrophages, may aggravate inflammation and adversely affect the local environment of implanted tissue engineered constructs upon internalization of MPs generated from these constructs as a consequence of implant degradation. The effects of MPs generated as byproducts of implant breakdown are exemplified by the internalization of wear debris from prostheses, such as hip implants, resulting in aseptic loosening (Lohmann et al., 2000; Vermes et al., 2000; Bainbridge et al., 2001), as well as enhancement of the immune response to cellular antigens released from tissue engineered constructs due to an adjuvant effect of the biomaterial component (Matzelle and Babensee, 2004; Yoshida and Babensee, 2004). Hence the understanding of molecular aspects of particle uptake by DCs and subsequent effect on the maturation state of DCs is paramount to the effective design of vaccines as well as tissue engineered devices.

In the study presented herein, the molecular aspects of phagocytosis of MPs by DCs, namely PLGA MPs, were investigated. The internalization of PLGA MPs by human monocyte-derived DCs was followed and visualized by using model fluorescent polystyrene latex microspheres and confirmed using coumarin 6-loaded PLGA MPs of approximately 3 μm in diameter. Orthogonal confocal laser scanning micrographs

(Figure 6-1) showed that 3 μm FYG microspheres and cPLGA MPs were indeed internalized by DCs, rather than associated on cell surface, implying that PLGA MPs used in our previous studies (Yoshida and Babensee, 2004), which were of similar size, were also phagocytosed by DCs. Association or co-localization of PLGA MPs with DCs has been previously observed using phase contrast microscopy of DCs in culture and light microscopy of Cytospin preparations, but internalization could not be confirmed with the assays used (Yoshida and Babensee, 2004).

Flow cytometry results corroborate the finding from with the confocal microscopy experiments that culturing DCs with either FYG microspheres or cPLGA MPs resulted in their internalization (Figure 6-2). Fluorescence peaks resulting from the association of FYG microspheres with DCs appeared as sharp distinct peaks, likely corresponding to the fluorescence intensity values proportional to the number of fluorospheres internalized by DCs; however, DCs cultured with cPLGA MPs exhibited one broader peak. This is likely due to the broader size distribution of PLGA MPs as compared to the FYG microspheres. Although both cPLGA MPs and FYG microspheres had a mean diameter of 3 μm, the range of diameter for PLGA MPs and FYG microspheres were approximately ± 3 μm and ± 0.5 μm, respectively (data not shown). It is speculated that with the wider range in size distribution, the correlation between the levels of fluorescence to the number of cPLGA MPs is not as close as that for FYG microspheres, resulting in a broader histogram spanning the FITC fluoresce spectrum.

The uptake of the fluorospheres by iDCs was decreased by incubation of iDCs at 4°C or by pretreatment with cytochalasin D (Figure 6-2), an inhibitor of actin polymerization including phagocytosis (Goddette and Frieden, 1986). Thus these results

imply that internalization of the fluorospheres is a dynamic process involving actin filaments. Moreover, the uptake of fluorospheres by DCs was trypsin-sensitive and dependent on extracellular calcium concentration, as pretreatment of iDCs with trypsin or EDTA, respectively, led to decreased fraction of DCs that were fluorosphere-positive (Figure 6-3). These results suggest that the uptake of fluorospheres by DCs is an active cellular process requiring Ca^{2+}, and likely receptor-mediated, as protease treatment can remove cell surface proteins, including endocytic receptors. It would be of great interest to determine the endocytic receptors involved in the fluorosphere uptake, in particular the uptake of PLGA MPs, as it relates to the PLGA-induced DC maturation and its mechanisms. Previous studies investigating receptors involved in DC nibbling showed that the treatment of monocyte-derived DCs with pronase, a mixture of proteases, resulted in decreased expression of several receptors including scavenger receptors, α_v-integrin, and C-type lectin receptors, as measured by flow cytometry (Harshyne et al., 2003). Similar studies to assess receptors involved in the internalization of PLGA MPs as well as the receptors involved in the subsequent DC maturation upon contact with PLGA MPs are currently underway.

Our previous study has shown that PLGA presented to DCs as MPs enhanced DC maturation, while PLGA presented as a flat surface did so to a lesser degree, supporting the notion that phagocytosis of particles may contribute to the biomaterial effects on DC maturation (Sallusto et al., 1995; Singh and O'Hagan, 1999). From the results presented herein using fluorospheres and pharmacological agents that inhibit cellular phagocytosis activity, it was speculated that internalization of PLGA MPs contributed to the maturation of DCs. However, culturing DCs with PLGA MPs of different sizes that were

either small enough or too large to be phagocytosed, with the same total surface area exposed to cells, revealed that while increasing the dose of the MPs increased the expression level of co-stimulatory and MHC molecules, the size of the MPs did not, suggesting that within the range tested, phagocytosis was not the main contributor in the PLGA MP-induced DC maturation. Moreover, as phenotypical changes were observed with exposure of DCs to PLGA films, and as there was a differential maturation effect depending on biomaterials used (manuscript in preparation; CHAPTER 7) (Babensee and Paranjpe, 2005), it is expected that DC maturation upon exposure to PLGA MPs is not solely due to phagocytosis. Further, the secretion of the autocrine factor, TNFα, was increased in a PLGA MP dose-dependent manner, which is in agreement with our previously reported results of PLGA MP-induced DC maturation, as measured by co-stimulatory and MHC molecule expression and allostimulatory capacity (Yoshida and Babensee, 2004). Recently, a study investigating the changes in phenotype and function of DCs upon loading with PLGA microspheres demonstrated that iDCs cultured in serum-free conditions phagocytosed many rhodamine-containing PLGA microspheres, in accord with our study, however, without increasing the expression of co-stimulatory and MHC class II molecules, nor TNFα secretion (Waeckerle-Men et al., 2004). The disparity between these two studies with regards to DC maturation upon loading with PLGA microspheres may be due to several factors. While the size of the PLGA MPs was similar, the composition of PLGA polymer used, methods for microsphere preparation, and the dose MPs to DCs were not the same between the two studies. More importantly, DC culturing conditions were different; in particular, in our studies, DCs were cultured in the presence of 10% FBS or 1% autologous plasma, while in the other study, DCs were

cultured in a serum-free environment. It is speculated that the presence of serum would lead to instantaneous adsorption of proteins onto the PLGA MP surface, and the subsequent effects of PLGA MPs may be directed in part by the adsorbed proteins, and that various factors in serum may have played a role in the maturation of human monocyte-derived DCs upon exposure to PLGA MPs. Similarly, culturing of murine monocytes or human blood monocytes with PLGA microspheres resulted in their activation, and opsonization of PLGA microspheres with different chemical end groups in serum resulted in increased phagocytosis of these PLGA particles, suggesting a role for protein adsorption on particle uptake (Prior et al., 2002). While the exact factors that affect the biomaterial particle phagocytosis remain elusive, the cytokines present in the microenvironment may play a role, as expression of co-stimulatory and MHC molecules upon uptake of polystyrene fluororescent microspheres of comparable size by murine bone marrow-derived DCs can be lowered by pretreatment of cells with IL-10, without affecting phagocytic capacity (Faulkner et al., 2000).

The experiments presented herein begin to address the role of phagocytosis on PLGA MP-induced DC maturation, and its associated adjuvant effect. While our previous study made an effort to address the differential effect of PLGA MPs and films on DC maturation, further studies are underway to determine the role of various receptors in internalization of different biomaterial MPs as well as in the subsequent DC maturation.

CHAPTER 7

DIFFERENTIAL EFFECTS OF AGAROSE AND POLY(LACTIC-*CO*-GLYCOLIC ACID) ON DENDRITIC CELL MATURATION*

INTRODUCTION:

The advent of innovative combination products has raised new regulatory concerns previously not considered. Some such combination products combine biomaterials with cells, DNA, or proteins, and include vaccine systems with non-viral polymeric carriers and tissue engineered constructs in which cells are delivered with a biomaterial scaffold. As the purpose of the biomaterial varies by application, identifying whether the biomaterial, due to its associated adjuvant effect, is intended or not to support the host immune response towards the product is essential in designing and choosing a material that is most appropriate for a given application. In tissue engineering applications, immune responses are to be minimized to avoid graft rejection whereas vaccine strategies aim to enhance the immune response to achieve protective immunity. Consequently, a biomaterial with high or low adjuvant effect, respectively, would be ideal for such applications.

While the host inflammatory response associated with biomaterials has been studied in great depth (reviewed in (Tang and Eaton, 1995; Babensee et al., 1998; Rihova, 2000)), the adjuvanticity of biomaterials in the context of tissue engineering and combination products is not as well characterized. For example, poly(lactic-*co*-glycolic acid) (PLGA), a biomaterial commonly studied in both tissue engineering and vaccine delivery applications, has been shown to act as an adjuvant. Delivery of a model antigen

* Modified from Yoshida M, Babensee JE. Differential effects of agarose and poly(lactic-*co*-glycolic acid) on dendritic cell maturation. Manuscript to be submitted to Journal of Leukocyte Biology.

with PLGA was shown to result in increased production of antibody against the model antigen (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001; Matzelle and Babensee, 2004), with the physical form of the PLGA affecting its adjuvant effect (Bennewitz and Babensee, 2005).

Moreover, because adjuvants act through the maturation of antigen presenting cells such as the dendritic cells (DCs) (Singh and O'Hagan, 1999), as characterized by increase in co-stimulatory and MHC molecule expression, cytokine secretion, and allostimulatory capacity (Cella et al., 1997; Banchereau and Steinman, 1998), the effect of PLGA on DC maturation has been studied with varying results, likely due to the differences in the DC culturing systems used (Sun et al., 2003; Waeckerle-Men et al., 2004; Yoshida and Babensee, 2004). Dendritic cells are professional antigen presenting cells central in the control of the immune response by connecting innate and adaptive immunity (Banchereau and Steinman, 1998; Banchereau et al., 2000). In the immature state, DCs reside in peripheral tissue and monitor the surrounding microenvironment by capturing and internalizing antigens (Banchereau and Steinman, 1998). Upon encountering a stimulus such as pathogen infection or "danger" signals, including byproducts of necrosis and inflammation or tissue damage, DCs undergo a maturation and present the previously processed antigens in the context of MHC molecules for activation of naïve T lymphocytes (Matzinger, 1994; Banchereau and Steinman, 1998; Gallucci et al., 1999). In addition to particulates that DCs may encounter endogenously, synthetic materials including particles of PLGA have been shown to be phagocytosed by DCs (CHAPTER6) (Lutsiak et al., 2002; Newman et al., 2002). The maturation of DCs result in functional and associated phenotypical changes, such as the decrease in the

expression of endocytic and adhesion receptors, increase in the expression of MHC and co-stimulatory molecules (Winzler et al., 1997; Banchereau and Steinman, 1998; Banchereau et al., 2000), and loss of phagocytic activity (Sallusto and Lanzavecchia, 1994).

Using human monocyte-derived DCs cultured in the presence of serum or autologous plasma, we have demonstrated that DCs treated with PLGA express higher levels of co-stimulatory and MHC molecules and are more allostimulatory, as compared to immature DCs, but to an extent lower than that induced by a lipopolysaccharide, a known stimulator of DC maturation (Yoshida and Babensee, 2004). Recently, differential effects of several biomaterials on DC maturation were reported, where some biomaterials induced increased DC expression of co-stimuulatory and MHC molecules as compared to immature DCs, whereas others did not (Yang et al., 2002; Babensee and Paranjpe, 2005). Such results suggest that inherent differences in the biomaterial itself, presumably its chemical composition and physical properties and consequent protein adsorption, can affect the state of DC maturation.

In this study, we investigated further the differential effects two model biomaterials, agarose and PLGA, on DC maturation. These particular biomaterials were chosen for comparison as PLGA has shown adjuvant effect (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001; Matzelle and Babensee, 2004), including DC maturation (Yoshida and Babensee, 2004) whereas agarose, while not studied with DCs, has been shown to induce minimal inflammatory response (Starke et al., 1987; Rahfoth et al., 1998). By comparing these two biomaterials, which seemingly have dissimilar adjuvant effect, we aimed to substantiate the differential biomaterial adjuvanticity, by

focusing on the maturation of DCs. Such differential biomaterial effects can be explored for the development and design of biomaterials suitable for each specific application, to accordingly modulate the host immune response.

METHODS:

Preparation of poly(lactic-co-glycolic acid) microparticles

Poly(lactic-*co*-glycolic acid) microparticles (MPs) were prepared by a single emulsion solvent evaporation technique adapted from a previously described method (Wake et al., 1998), reported elsewhere (Yoshida and Babensee, 2004). Briefly, MPs of approximately 3 μm in diameter were formed by homogenizing dichloromethane (DCM) containing 2.5% w/v PLGA (molar ratio: 75:25, inherent viscosity: 0.69 dL/g in trichloromethane) (Birmingham Polymers, Birmingham, AL) with 0.3% v/v aqueous poly(vinyl alcohol) (88% hydrolyzed) (PVA) (Sigma, St. Louis, MO) at 9000 rpm. Immediately after homogenization, 2% (v/v) aqueous isopropanol (Sigma) was added to initiate precipitation, and the mixture stirred overnight at room temperature to evaporate the solvent. Resulting MPs were collected by centrifugation at 1000 rpm for 10 minutes, washed twice in 2% isopropanol, and three times in distilled deionized H_2O (ddH_2O). Microparticles were resuspended in ddH_2O and their size distribution characterized using a Coulter Multisizer II (Coulter Corporation, Miami, FL). Prior to use, MPs were UV sterilized for 1 hr, and stored at 4°C. Endotoxin content of PLGA MP suspension was determined by chromogenic Limulus Amebocyte Lysate assay (QCL-1000 Chromogenic LAL Endpoint Assay, Cambrex, Walkersville, MD), and was below limits of detection (absorbance readings below blank sample).

Larger MPs of approximately 30 μm in diameter were prepared in a similar manner, with a lower homogenization speed of approximately 950 rpm. Upon washing with isopropanol as described above, MPs were successively filtered using cell strainers with mesh size of 70 μm, followed by a 40 μm strainer (Becton Dickinson, Franklin Lakes, NJ). Resulting filtrate was filtered again using a 20 μm nylon mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA). After this filtration, MPs in the membrane retentate were collected by washing the mesh with at least 50 ml ddH_2O. Resulting MP suspension was washed 3 times with ddH_2O, and UV sterilized and stored at 4°C as described above. Endotoxin content was below limits of detection (absorbance readings below blank sample).

Preparation of poly(lactic-co-glycolic acid) film

Poly(lactic-*co*-glycolic acid) films were fabricated using a casting technique without a porogen (Ishaug et al., 1997), as previously reported (Yoshida and Babensee, 2004). Briefly, 10% w/v 75:25 PLGA was dissolved in DCM overnight at room temperature, and poured onto a 50 mm or 100 mm Teflon Petri dish (Cole-Parmer, Vernon Hills, Illinois). Upon evaporation of the solvent and drying, the film was punched into an appropriate size, and washed for 1 hr in ddH_2O, changing water every 15 min. The film was dried 30 min per side in a sterile laminar flow hood and UV sterilized for 30 min per side prior to use. Endotoxin assay was performed on a smaller film of 4.5 mm in diameter, and determined to be below limits of detection.

Preparation of agarose microparticles

Microparticles of polystyrene coated with agarose (agarose MPs), of 2 μm and 30 μm in diameter, were purchased from Micromod (Micromod, Rostock-Warnemuende, Germany). Prior to use, the MPs were washed three times in 70% ethanol and three times in ddH_2O. Contribution of 2 μm and 30 μm MPs suspension to cell culture endotoxin content was 0.0045 EU/ ml and 0.00106 EU/ ml at 1:1 MP: cell ratio, respectively. Previous study has shown that minimum E. coli endotoxin concentration of 100 EU/ mL was required for DC maturation (Jotwani et al., 2003).

Preparation of agarose film

Agarose (type V; sulfate content of ≤ 0.30%, gel strength of ≥800 g/ cm^2 at 1.0%, and gel point of 42°C±1.5°C at 1.5%) was purchased from Sigma. This particular agarose was chosen as it had a high gel point, enabling it to remain in the hydrated gelled state at temperature of 37°C, temperature used for cell culture. Aqueous solution of 3% w/v agarose in ddH_2O was prepared and heated using a microwave until boiling and visible homogeneity was reached. The film was prepared by dispensing 1 ml of agarose solution into a well of a 6 well plate (Corning, Corning, NY), and allowed to solidify at a temperature of 4°C for at least 30 min, and brought back to room temperature for another 30 min prior to use. Endotoxin content of 2 batches of agarose films were measured, and were determined to be 0.098 EU/ ml and 0.17 EU/ ml, respectively for a film of 5.6 mm in diameter.

Dendritic cell culture

Human blood was obtained from volunteers with informed consent, according to a protocol approved by the Institutional Review Board of Georgia Institute of Technology, #00A018. Dendritic cells were derived from human peripheral blood mononuclear cells (PBMCs) using a previously described method (Romani et al., 1996) with some modifications, as previously reported (Yoshida and Babensee, 2004). Briefly, PBMCs were isolated from blood by differential centrifugation using Histopaque (Sigma). Upon lysing erythrocytes and washing, PBMCs were resuspended at a concentration of $5x10^6$ cells/ ml in RPMI-1640 containing 25 mM HEPES and L-glutamine (Gibco BRL, Grand Island, NY) with 100 U/ ml penicillin-streptomycin (Cellgro, Herndon, VA) and 10% (v/v) heat inactivated filter sterilized fetal bovine serum (Invitrogen, Carlsbad, CA) (DC media). Cells were plated in a volume of 10 ml/ plate in a 100x20 mm tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and incubated for 2 hrs to select for adherent monocytes. After the incubation, plates were washed 3 times with warm DC media to remove non-adherent cells, and the remaining adherent cells were supplied with 10 ml DC media and incubated with 2000 U/ ml GM-CSF and 1600 U/ ml IL-4 (Peprotech, Rocky Hill, NJ). On day 5 of culture, loosely- and non-adherent cells containing DCs were collected by centrifugation for 10 min at 1100 rpm, resuspended in DC media with 1000 U/ ml GM-CSF and 800 U/ ml IL-4, and plated in a 6 well plate (Corning, Corning, NY) at a concentration of $5x10^5$ cells/ well in 3 ml with or without stimulants. For the induction of mature DCs (mDCs), 1 μg/ ml lipopolysaccharide (LPS) (E. Coli 055:B5) (Sigma) was added, and cells with no additional maturation stimulants were used as iDCs. The effect of treatment of iDCs with biomaterial MPs or films was

tested by adding MP suspension at indicated MP:cell ratios or by plating cells onto a PLGA or agarose film. After 24 hrs of DC treatment with the biomaterial, DCs were collected for further analysis, and cell culture supernatant was collected, cleared by centrifugation, and stored at -20°C until analysis.

Cell morphology

Morphology was monitored throughout the duration of culture by phase contrast microscopy of cells in culture. Dendritic cells treated with biomaterials were processed for Cytospin preparations (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburgh, PA), stained with Differential Hematology Stain (Astral Diagnostics, West Deptford, NJ), and analyzed by light microscopy.

Number of MPs ingested by DCs was determined by counting the number of MPs associated with DCs from 6 different Cytospin preparations from 6 separate experiments.

Cell surface marker expression

Levels of surface marker expression were monitored throughout the culture using by flow cytometry as previously described (Yoshida and Babensee, 2004). Dendritic cells were stained with mouse anti-human monoclonal antibody against CD40 (clone B-B20; IgG1κ), CD80 (clone BB1; IgMκ), CD86 (clone BU63; IgG1κ) (Southern Biotech, Birmingham, AL), CD83 (clone HB15a; IgG2b) (IO Test Immunotech Beckman Coulter, Marseille, France), HLA-DQ (clone TU169; IgG2aκ), or HLA-DR (clone TU36; IgG2aκ) (Becton Dickinson Pharmingen, San Diego, CA) and analyzed using BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using

WinMDI 2.8 (Scripps Research Institute, La Jolla, CA) and BD FACS DiVa Option 2.0 (Becton Dickinson Biosciences, San Jose, CA).

Tumor Necrosis Factor α (TNFα) ELISA

The amount of TNFα produced by DCs in the cell culture supernatant was analyzed by ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's directions. Cell culture supernatant was cleared by centrifugation and stored at -20°C until analysis. Dendritic cells recovered from each treatment groups were collected, counted, and viability assessed. In addition, DNA content was analyzed for each treatment group using picoGreen dsDNA quantification kit (Invitrogen) per manufactuer's directions and amount of TNFα secreted reported per μg DNA.

Mixed lymphocyte reaction (MLR)

The effect of biomaterials in inducing DCs to become efficient antigen presenting cells was measured by their allostimulatory capacity of DCs to stimulate T cell proliferation was assessed using an allogeneic MLR as previously described (Yoshida and Babensee, 2004). On day 5 of DC culture, allogeneic T cells were isolated from PBMCs by negative selection using Pan T cell magnetic isolation (Miltenyi Biotech, Auburn, CA) according to manufacturer's protocols, and used as responder cells. T cells were resuspended in RPMI-1640 with 25 mM HEPES and L-glutamine (Gibco BRL) with 100 U/ ml penicillin-streptomycin (Cellgro) and heat inactivated filter sterilized 10% (v/v) human AB serum (Biowhittaker, Walkersville, MD) (complete RPMI-10 media) and plated at a concentration of $1x10^5$ cells/ well in a 96 well flat-bottomed plate

(Corning). Dendritic cells treated or not with biomaterials or LPS were resuspended at $1.6x10^5$ cells/ ml, and treated with 25 μg/ ml mitomycin C (Sigma) for 30 minutes to prevent their proliferation. Upon extensive washing with complete RPMI-10 media, DCs were resuspended in complete RPMI-10 media and added to responder cells in triplicates at graded DC: T cell ratios. Cells were co-cultured for 4 days, and the DC-induced T cell proliferation was measured using BrdU incorporation by colorimetric ELISA (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions.

Preparation of DC nuclear extract and measurement of activity of NFκB family of transcription factors

Nuclear extracts from differentially treated DCs were prepared using TransFactor Extraction kit (Becton Dickinson Clontech, Palo Alto, CA) according to manufacturer's directions. Briefly, DCs were collected and washed twice with ice cold PBS by centrifugation at 450 x g for 5 min at 4°C. The cell pellet was resuspended in lysis buffer containing protease inhibitors and allowed to incubate for 15 min on ice. After incubation, the suspension was centrifuged, and the particulate fraction was resuspended in lysis buffer. The cells were disrupted by forcing the suspension through a 27 gauge needle. The resulting suspension was centrifuged at 11,000 x g for 20 min at 4°C. The supernatant and the pellet from this centrifugation were considered to be cytosolic and nuclear fractions, respectively. The pellet containing the cell nuclei was resuspended in an extraction buffer containing protease inhibitors, and nuclear membrane disrupted by forcing the suspension through at 27 gauge needle. The suspension was centrifuged at 21,000 x g for 5 min at 4°C, and the supernatant from this centrifugation was considered

to be the nuclear extract, transferred to a chilled new tube, and stored at -70°C until analysis.

Activities of members of NFκB family of nuclear transcription factor were assessed using TransFactor NFκB Family kit (Becton Dickinson Clontech), an ELISA-based method of detecting transcription factor activities, per manufacturer's protocol. In brief, nuclear extracts were incubated for 60 min in a well pre-coated with consensus binding sequence. Upon washing, primary antibodies corresponding to each member of NFκB family was added, and allowed to incubate for 60 min. The plate was washed, and incubated for additional 30 min with the secondary antibody. The detection of binding was measured by the addition of tetramethylbenzidine substrate, and measuring the colorimetric development at 655 nm.

Statistical Analysis

Statistical analysis was performed using general linear model ANOVA with Minitab software (Version 13.20, Minitab Inc., State College, PA), and pairwise comparison to appropriate control group as indicated was made, unless otherwise indicated, with *p*-values of less than or equal to 0.05 considered to be significant.

RESULTS:

Dendritic cells treated with agarose or PLGA microparticles or films exhibit similar morphology

Immature DCs (iDCs) derived from PBMCs were cultured either with agarose or PLGA MPs or on films, and their morphology was assessed by phase contrast microscopy and Cytospin preparations. Phase contrast microscopy of cells in culture revealed that culturing peripheral blood monocytes with GM-CSF and IL-4 resulted in the detachment of cells from the culture plate and formation of cell clusters, hallmarks of DC development; in particular, DCs cultured on agarose films showed most detachment and formation of visible cellular clumps, likely due to the hydrophilic nature of the agarose film on which the cells were plated (data not shown). Dendritic cells treated with the biomaterial MPs or films as well as those treated with LPS to yield mature DCs (mDCs) as positive control exhibited dendrite processes to similar extent (Figure 7-1). Microparticles of PLGA or agarose were associated with DCs, and were likely phagocytosed. Internalization of PLGA MPs has been previously confirmed by confocal microscopy of DCs cultured with PLGA MPs containing fluorescent dye (CHAPTER 6).

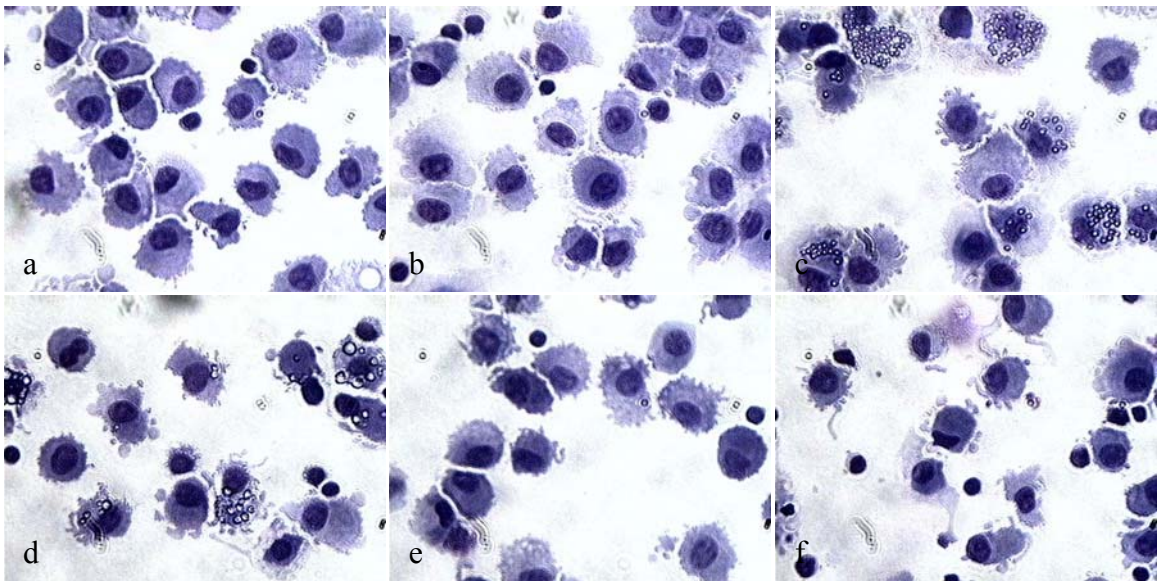

Figure 7-1: Dendritic cells treated with agarose or PLGA MPs or films exhibit similar morphology.
Immature DCs derived from peripheral blood monocytes in the presence of GM-CSF and IL- 4 (a), cultured with agarose MPs (c) or film (e) or PLGA MPs (d) or film (f) showed similar morphology to that of DCs matured by LPS (b), with the presence of dendritic processes. Agarose and PLGA MPs used to treat DCs are associated with the cells. Original magnification: 40x.

Dendritic cells treated with PLGA film express higher levels of co-stimulatory and MHC molecules than DCs treated with agarose film

As a measure of maturation, DCs treated with biomaterial MPs or films were analyzed by flow cytometry for their surface expression of co-stimulatory and MHC molecules. Co-stimulatory molecules such as CD40, CD80, and CD86, and MHC class II molecules such as HLA-DQ and HLA-DR, as well as CD83, increase upon DC maturation (Zhou and Tedder, 1995; Banchereau and Steinman, 1998). Dendritic cells treated with PLGA films increased the expression levels of markers evaluated, whereas DCs treated with agarose film did not, or did so to a lesser extent (Figure 7-2a). In particular, higher expression of CD80 and CD86 as measured as fold increase above iDC levels was induced by PLGA film treatment as compared to agarose film treatment at a statistically significant level (Figure 7-2b). Similarly, DCs cells treated with PLGA MPs were previously shown to increase the expression of these molecules (Yoshida and Babensee, 2004) while those treated with agarose MPs at the same MP: cell ratio did not alter the expression of any of these markers (Figure 7-3).

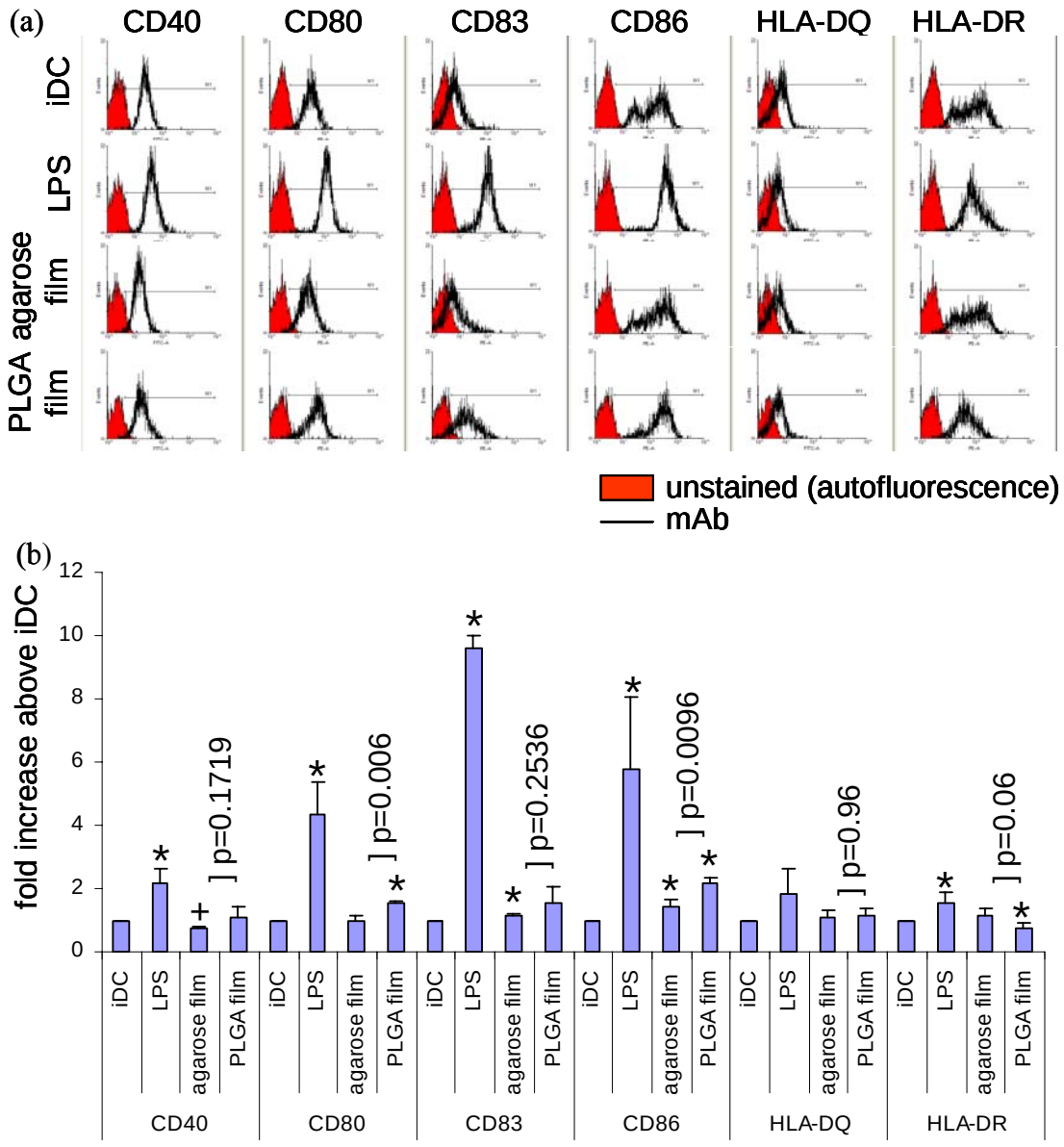

Figure 7-2: Dendritic cells treated with PLGA express higher levels of co-stimulatory and MHC molecules than DCs treated with agarose.
Expression levels of co-stimulatory and MHC molecules of DCs treated with PLGA or agarose films were measured using flow cytometry. Dendritic cells treated with PLGA film expressed higher levels of CD80, CD86, and HLA-DR than DCs cultured on agarose film. This experiment was repeated three times with similar results, and representative results are shown (a). Fold increase in gMFI values for each treatment group as compared to that of iDC control corroborate enhanced marker expression; PLGA-treated DCs showed higher fold increase in marker expression than did agarose-treated DCs at a statistically significant level (b). mean±SD, n=3. */+: $p<0.05$ compared to iDC (above/ below iDC, respectively). Brackets indicate comparison between agarose and PLGA films with *p*-values indicated.

Microparticle dose, but not size, affects PLGA MP-induced DC maturation

To determine whether the phagocytosis of MPs contributed to PLGA-induced DC maturation previously observed, DCs were cultured with PLGA or agarose MPs of phagocytosable (2 μm) or non-phagocytosable (20 μm or 30 μm) sizes while maintaining constant exposed surface area. This approach of assessing the role of phagocytosis on DC maturation was chosen as use of phagocytosis and macropinocytosis inhibitors cytochalasin D or amiloride alone increased expression of several co-stimulatory markers in our hands (Figure A2, APPENDIX). Use of MPs of differing sizes with constant biomaterial surface area provided a cleaner system in which to assess the role of biomaterial MP phagocytosis on DC maturation.

For DCs treated with PLGA MPs, the expression levels of co-stimulatory and MHC class II molecules increased in a MP-dose dependent manner, but were not affected by the MP size (Figure 7-3a). In contrast, the expression levels of markers by DCs treated with agarose MPs were not increased above that of iDCs regardless of MP dose or size, within the range tested (Figure 7-3a). In addition, significant fold increase in marker expression was induced by treatment of DCs with PLGA MP at both sizes and doses, whereas with agarose MPs, only 30 μm MPs at the higher dose increased CD80 exprssion (Figure 7-3b). While the difference in size of PLGA MPs did not alter the expression of maturation markers, at the higher dose, treatment with large PLGA MPs resulted in slightly higher expression of CD83 than treatment with smaller PLGA MPs (Figure 7-3b).

To ascertain that this difference in MP-induced DC maturation was not due to the difference in the number of phagocytosed MPs, distribution of MPs ingested by DCs were tabulated based on Cytospin preparations (Table 4). For both agarose and PLGA MP-treated cells, over 70% of the DCs ingested fewer than 5 MPs, with agarose MP-treated DCs ingesting a higher number of MPs.

Table 4: Fraction of DCs with phagocytosed 2 μm MPs

MPs ingested	0	1-5	6-10	11-15	16-20	>20
agarose	27.0%	44.6%	13.3%	5.9%	2.1%	7.0%
PLGA	40.0%	53.5%	6.5%	0.0%	0.0%	0.0%

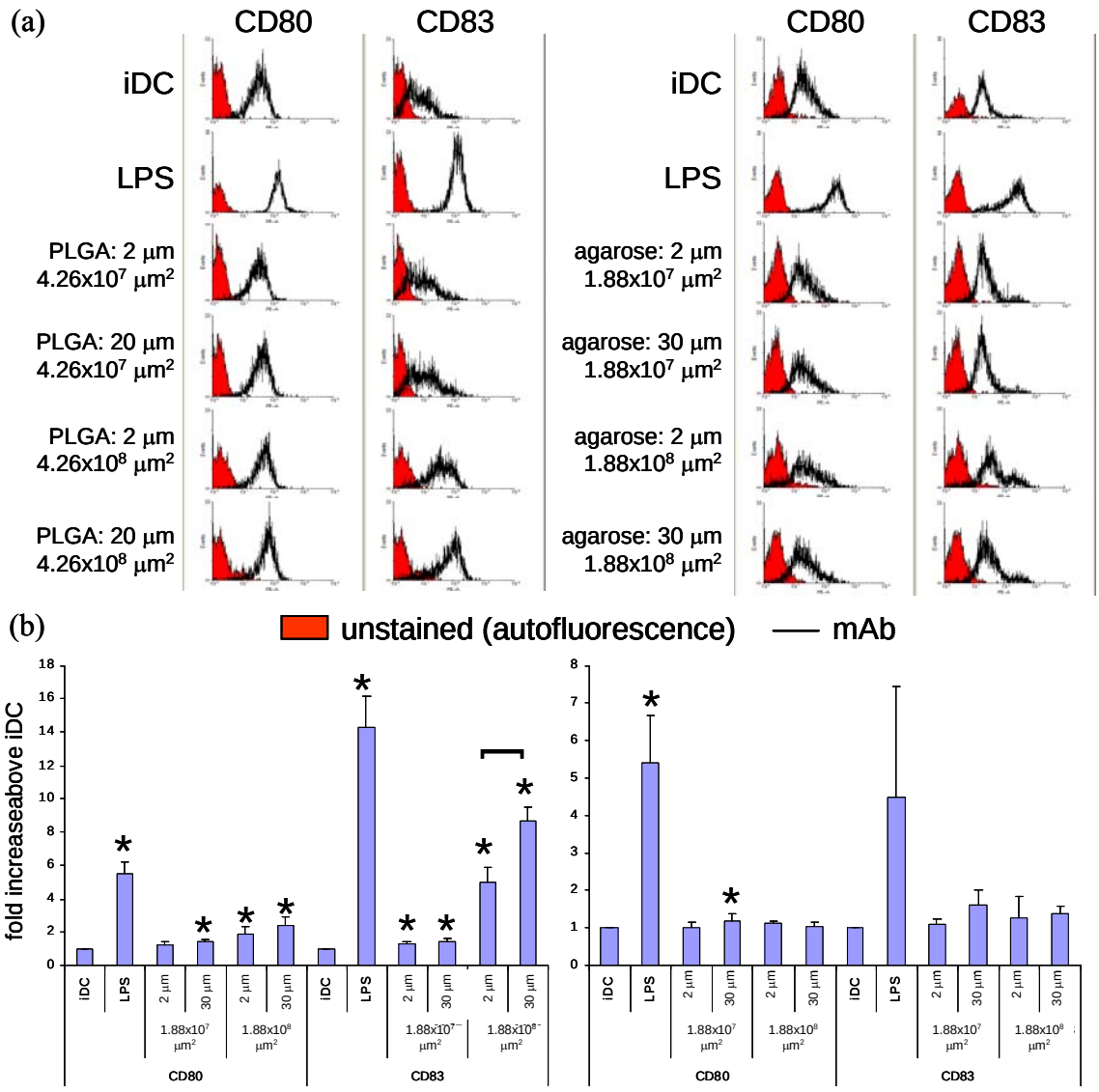

Figure 7-3: Dose of MPs, but not size, affects PLGA MP-induced DC maturation. Dendritic cells were treated with PLGA or agarose MPs of different sizes (small MPs: 2 μm, large MPs: 20 μm or 30 μm) at two different doses. Cells exposed to the same biomaterial surface area (same dose) but with different MP sizes showed same level of CD80 and CD83 expression for both agarose and PLGA MPs. Increasing the dose of PLGA MPs resulted in increased expression of CD80 and CD83, whereas increasing the dose of agarose MPs did not. This experiment was repeated three times with similar results, and representative results are shown (a). Fold increase in gMFI values for each treatment group as compared to that of iDC control show that treatment of DCs with PLGA but not agarose MPs at both sizes and doses induce increased expression of CD80 and CD83; additionally, for PLGA MPs of both sizes, increasing the dose of MPs also increased the fold increase of marker expression (b). mean±SD, n=3. *: $p<0.05$ compared to iDC. Bracket indicates statistically significant difference between MP size difference at a given MP dose.

Dendritic cells treated with PLGA are more allostimulatory than DCs treated with agarose in a mixed lymphocyte reaction (MLR)

To evaluate the maturation functionality of DCs treated with PLGA or agarose as antigen presenting cells, differentially treated DCs were co-cultured with allogeneic T cells and the proliferation of T cells measured in an MLR. Dendritic cells treated with PLGA, in particular those treated with MPs, induced proliferation of allogeneic T cells, whereas those treated with agarose did not (Figure 7-4). In a direct comparison of DCs treated with agarose or PLGA films, cells treated with PLGA films induced proliferation of allogeneic T cells higher than did DCs treated with agarose film (data not shown).

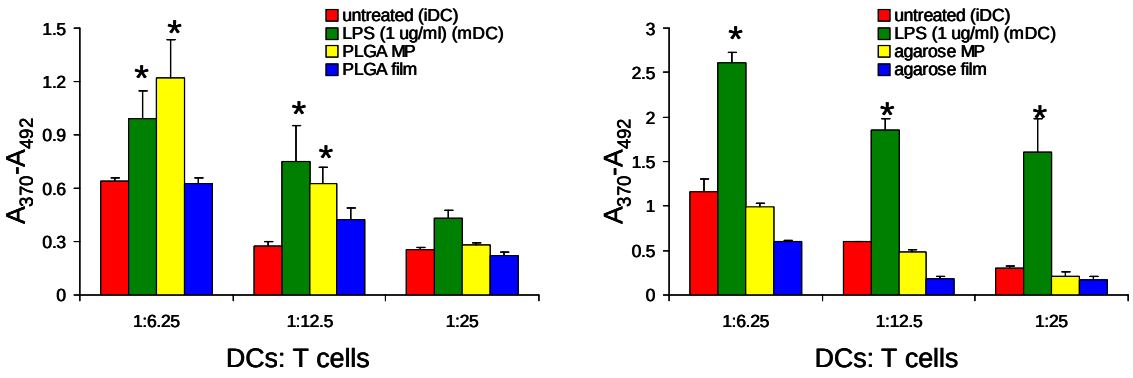

Figure 7-4: Dendritic cells treated with PLGA are more allostimulatory than DCs treated with agarose in a mixed lymphocyte reaction (MLR).
Differentially treated DCs were co-cultured for 4 days with allogeneic T cell to measure their allostimulatory capacity. The proliferation of T cells was measured using BrdU incorporation. Dendritic cells treated with PLGA MPs were allostimulatory whereas those treated with agarose MPs or films were not. This experiment was repeated three times with various responder: stimulator cell donors pairs with similar results, and representative results are shown. mean±SD, n=3 wells, *: $p<0.05$ compared to iDC.

Dendritic cells treated with PLGA film secrete more TNFα than DCs treated with agarose film

As another functional evaluation of DC maturation, amounts of TNFα secreted in the cell culture supernatant of differentially treated DCs were measured. Cell culture supernatants from iDCs left untreated, treated with PLGA or agarose films, or matured with LPS were collected, cleared by centrifugation, and stored at -20°C until analysis by ELISA. For each treatment group, DCs were retrieved, and assessed for viability and DNA content, and amount of TNFα secreted by DCs reported based on DNA content to account for any variations in cell count or DNA content due to treatment. As expected, iDCs and LPS-matured DCs secreted very low or high amounts of TNFα, respectively, suggestive of their maturation state (Figure 7-5). While not as high as LPS-induced DC secretion, PLGA films treatment induced secretion of TNFα above that released by iDCs, with PLGA-treated DCs secreting more TNFα than agarose-treated DCs.

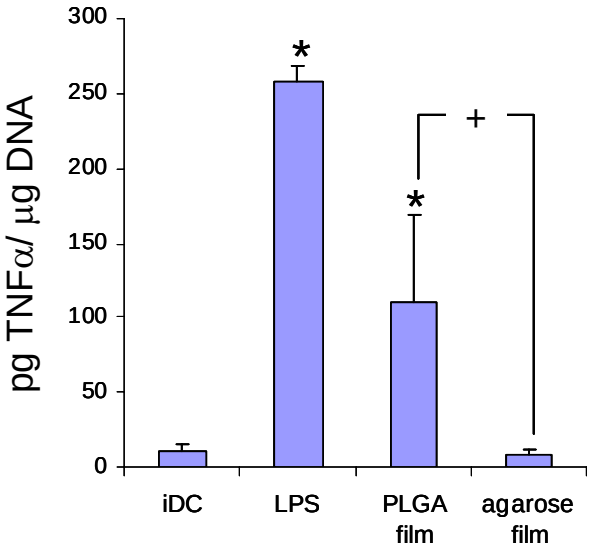

Figure 7-5: Dendritic cells treated with PLGA film secrete more TNFα than DCs treated with agarose film.
Cell culture supernatants from iDCs left untreated or treated with PLGA film, agarose film, or LPS were assessed for level of TNFα. Immature DCs and agarose film-treated DCs did not produce appreciable levels of TNFα whereas LPS-matured DCs and PLGA film-treated DCs produced a significantly higher amount of the cytokine. Dendritic cells treated with PLGA film secreted higher amounts of TNFα than did DCs treated with agarose film. mean±SD, n=3. *: $p<0.05$ compared to iDC, +: $p<0.05$ between PLGA and agarose film-treated DCs.

Dendritic cells treated with agarose film show higher activation of NFκB family members than DCs treated with PLGA film

Activation of NFκB, family of a transcription factors composed of homo- and heterodimers of p50, p52, p65/RelA, RelB, and cRel, is one of the signaling pathways that controls genes involved in inflammatory and immune responses (Baeuerle and Henkel, 1994; Ghosh et al., 1998). In addition, NFκB has been shown to play a role in the regulation of DC maturation, including increased expression of co-stimulatory and MHC class II molecules and cytokine production (Rescigno et al., 1998; Ouazz et al., 2002). To begin to address the mechanism of biomaterial-induced DC maturation, activation levels of members of NFκB family in differentially treated DCs were evaluated. Dendritic cells treated with agarose or PLGA film showed levels of NFκB activation in between those of iDCs and LPS-matured DCs (Figure 7-6). However, DCs treated with agarose showed higher activation levels of all members of NFκB family assessed, as compared to PLGA-treated DCs.

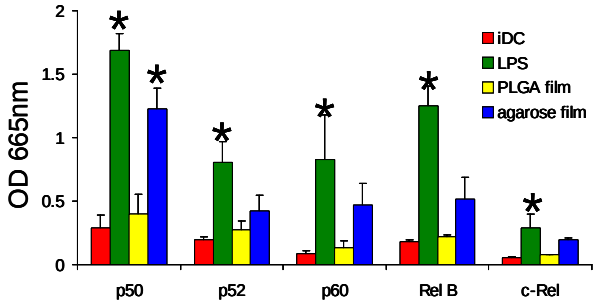

Figure 7-6: Dendritic cells treated with agarose film show higher activation of NFκB than DCs treated with PLGA film after 24 hrs of treatment.
Activation of members of NFκB family of nuclear transcription factor was measured in the nuclear extracts from differentially treated DCs. LPS- matured DCs showed significantly high activation of all members of the family evaluated. Treatment of DCs with agarose or PLGA films resulted in higher activation of NFκB family members as compared to iDCs, with agarose treatment resulting in higher activation. mean ± SD, n=3. *: $p<0.05$ compared to iDC.

DISCUSSION:

Use of biomaterials in combination products in which the material component is delivered together with a biological component requires the understanding of biomaterial adjuvant effect in potentiating the immune response against biological component of the product. The desired level of biomaterial adjuvanticity varies by application and thus a clear definition for the role of the biomaterial in supporting or not supporting the immune response towards the product is fundamental in effective design and selection of a material that is most suitable for a given application.

In this study, the adjuvant effect of two model biomaterials, agarose and PLGA, as measured by phenotypes of DC maturation was compared. We have previously shown that PLGA acts as an adjuvant in enhancing the humoral response against a co-delivered model antigen (Matzelle and Babensee, 2004; Bennewitz and Babensee, 2005), and that this adjuvant effect, at least in part, was explained by the induced maturation of DCs when treated with PLGA MPs or film (Yoshida and Babensee, 2004). These results led to the study of differential effects of biomaterials on DC maturation, specifically, the comparison of the effect of PLGA on DC maturation with a biomaterial commonly used in tissue engineering applications that has been shown to provoke little inflammation to provide a basis for the differential effects of biomaterial adjuvant effect. For this reason, agarose was chosen as a model biomaterial for comparison with PLGA. We have observed in this study that PLGA presented higher adjuvant effect as measured by many of the phenotypes associated with mature DCs. In particular, DCs treated with PLGA MPs or films showed higher expression of co-stimulatory and MHC molecules, allostimulatory capacity, and cytokine production, as compared to DCs treated with

agarose MPs or films. Further, DC maturation induced by PLGA MPs was shown to be independent of phagocytosis, at least in the capacity evaluated, as treating DCs with MPs of unphagocytosable size with same exposed surface area resulted in the same level of marker expression as compared to DCs treated with phagocytosable MPs. In contrast, agarose-treated DCs consistently showed higher activation of all members of NFκB family evaluated. While the degree of maturation as measured by DC phenotypes varied amongst donors in our hands, the trends were consistent. The induction of DC maturation by PLGA is unlikely due to endotoxin contamination, as PLGA MPs and films showed below detectable levels of endotoxin. In contrast, agarose MPs and films had measurable amounts of endotoxin present, but likely in a dose lower than that necessary to induce DC maturation, substantiating the significance of PLGA-induced DC maturation.

The differential expression of maturation markers by DCs treated with agarose or PLGA (Figure 7-2) is in accord with previously published results (Babensee and Paranjpe, 2005) and with other assessment of maturation including allostimulatory capacity (Figure 7-4) and TNFα production (Figure 7-5). The amount of TNFα measured in this study presented here is reported based on cellular DNA content, to account for any effect of treatment on cell viability. The viability of cells upon treatment was assessed, and was not different amongst the treatment groups, and was always higher than 97% (data not shown).

Cell culture supernatants from DCs treated with PLGA or agarose MPs collected 24 hrs post-treatment in separate experiments, although not in direct comparison, was measured by Cytometric Bead Array Human Inflammation kit (Becton Dickinson

Pharmingen). These results also showed that DCs treated with PLGA secreted higher amounts of cytokines (IL-12p70, IL-1, IL-6, TNFα, IL-8, IL-10) than those secreted by agarose treated DCs (Figure A3, APPENDIX). In general, the cytokine profile trends of DCs treated by agarose or PLGA were similar, with increasing concentrations of inflammatory cytokines, TNFα, IL-8 and IL-6 in a MP-dose dependent manner, while IL-12p70 was detected at a very low level, comparable to that released by iDCs. Secretion of IL-10 by DCs treated with agarose or PLGA was measurable but low, and increased with PLGA but not agarose MP dose. Dendritic cells treated with agarose or PLGA MPs secreted low amounts of IL-1β, which did not correlate with MP dose. While no direct comparison of agarose- and PLGA-treated DCs was made in the measurement of these cytokines, higher levels of TNFα, IL-8, and IL-6 induced suggest a Th1 skewed response of biomaterial-treated DCs. This result is in agreement with another study where the delivery of a model antigen with PLGA MPs resulted in a Th1 response as measured by production of IgG2 isotype antibody against the antigen (Newman et al., 1998). In our experiments, production levels of both IL-10 and IL-12p70 by DCs treated with agarose or PLGA were low. As IL-12 production is upregulated by MHC class II and CD40 ligation, and is downregulated by Th2 cytokine such as IL-10 (Koch et al., 1996), opposite trends in the levels of these two cytokines would have been more revealing of the Th polarization induced by the biomaterial treatment of DCs. A thorough analysis of time course of cytokine production as well as mRNA levels of these cytokines may help provide additional information on this aspect of biomaterial-induced DC maturation. The elucidation of differential cytokine profiles produced by biomaterials, and the types of T cell response induced by DCs treated with such biomaterials can guide the development

of novel biomaterials that can appropriately influence Th polarization for controlled host tolerance of tissue engineered grafts or combination products or protective immunity.

The lack of maturation effect observed on DCs treated with agarose is not surprising given that it elicits minimal inflammation upon implantation (Starke et al., 1987; Cadic-Amadeuf et al., 1992; Rollwagen et al., 1993; Spargo et al., 1994; Rahfoth et al., 1998; van Heeckeren et al., 2000). There are several differences between agarose and PLGA to which the differential DC maturation effects can be attributed. The first and foremost is the difference in the chemical composition of the two biomaterials. Agarose is a natural polysaccharide-derived from seaweed, composed of repeating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl units. Its gellation properties are thermoreversible due to hydrogen bond formation, facilitated by alignment of the agarobiose molecules (Shoichet et al., 1996). In contrast, PLGA is a synthetic polyester composed of variable molar ratios of lactic and glycolic acid, degradation products of PLGA which can be metabolized and excreted (Athanasiou et al., 1996). In the physical forms of MPs and films investigated in this study, films provide a more direct comparison of the materials, as they were made without any reagents other than the material itself and the solvent. While the weight per volume fractions of PLGA and agarose in their solvents were 10% and 3%, respectively, X-ray photon spectroscopy analysis of surface chemistry of the films showed theoretical compositions of carbon environment, as measured by percent carbon bonding in the three forms of C-C/H, C-O, or O-C=O (Figures 4-2c, 4-5b). The hydrophobicity of PLGA as compared to agarose implies that PLGA film likely adsorbed more proteins present in the cell culture, which may facilitate the interaction between the film and the DCs and the observed maturation.

Currently in our laboratory, efforts are being made to correlate adsorbed proteins on surfaces with self-assembled monolayers presenting methyl (CH_3), amine (NH_2), hydroxyl (OH), or carboxyl (COOH) terminal groups. In particular, quantification and identification of carbohydrate modifications of these adsorbed proteins and their effects on DC maturation via recognition by lectin receptors is under investigation.

While almost all of our assessment of differential effects of agarose and PLGA indicated that PLGA induced higher degree of DC maturation, agarose-treated DCs consistently showed higher activation of NFκB. NFκB family of transcription factors are composed of homo- and heterodimers of five proteins (p50, p52, p65/RelA, RelB, cRel), and regulates many of the genes involved in inflammatory and immune responses (Baeuerle and Henkel, 1994; Ghosh et al., 1998). Activation of NFκB pathway is involved in LPS- and bacteria-induced DC maturation, and is independent of MAPK activation (Rescigno et al., 1998; Rescigno et al., 1999; Arrighi et al., 2001). Similarly, recent studies have shown that polyunsaturated fatty acids can block the maturation of DCs and function independently of NFκB activation, suggesting another mechanism by which DCs can be matured independent of NFκB (Zeyda et al., 2005). The existence of DC maturation pathways independent of NFκB suggests that PLGA may induce DC maturation in such manner. Other studies have demonstrated that DCs lacking particular members of NFκB family, namely p50, RelA (p65), or cRel exhibited impaired DNA binding activity with no obvious defects in expression of MHC class I and II molecules as well as CD80 or CD86, or defects in response to LPS or TNFα (Ouazz et al., 2002), suggesting a disparity between co-stimulatory and MHC molecule expression and NFκB activation, as observed in our study.

In investigations of biomaterial-induced NFκB activation, hyaluronic acid and chondroitin sulphate A, both of which are naturally occurring glycosaminoglycans, have been shown to be increase DC maturation and NFκB activation (Yang et al., 2002). More interestingly, HeLa cells cultured on lipid films and hydrophilic surfaces exhibited different time course of NFκB activation (Kishida et al., 2001). Cells cultured on lipid and hydrophilic surfaces reached comparable levels of activation, but exhibited different time course of activation; HeLa cells cultured on lipid film reached peak activation after 15 min of cell culture which was reduced by 24 hrs post-treatment, whereas cells cultured on hydrophilic surfaces of polyacrylamide grafted with polyethylene or cellulose showed maximal activation at 24 hrs. A similar phenomenon may have occurred with PLGA and agarose treated DCs. In our study, NFκB activation was measured 24 hrs after the treatment with the biomaterial; this time point may have been optimal for hydrophilic agarose-treated DCs but too long to observe any activation of NFκB in hydrophobic PLGA-treated DCs which may have already peaked and declined.

Studies presented herein begin to address the differential effects of biomaterials on DC maturation, and the associated adjuvant effect and immune and inflammatory responses. While most assessments as measured by DC maturation indicated that PLGA produced higher adjuvant effect than did agarose, the incongruence of NFκB activation underscores the need to evaluate various aspects of material biocompatibility and its effect on cell behavior to fully appreciate the complex events that occur *in vivo* when products containing such biomaterials are implanted and encountered by the host defense mechanisms. Elucidation of differential biomaterial adjuvant effect and the material physicochemical properties which influence the adjuvanticity can help guide the

development of novel materials and define design and selection criteria for biomaterials for various applications including vaccine strategies and regenerative medicine.

CHAPTER 8

ROLE OF TOLL-LIKE RECEPTORS IN POLY(LACTIC-*CO*-GLYCOLIC ACID) INDUCED DENDRITIC CELL MATURATION

INTRODUCTION:

The development of combination products such as tissue engineered constructs which combine biomaterials with biological components have prompted the understanding for the role of the biomaterial component in the host immune response encountered upon implantation. Depending on the application of the device, the purpose of the biological component may be to induce immunogenicity, as in the case of vaccines, or to aid in tissue regeneration, as in the case of tissue engineering. While inflammation associated with biomaterial implantation in the context of tissue engineering has gained much focus (reviewed in (Tang and Eaton, 1995; Babensee et al., 1998; Rihova, 2000)), the effect of biomaterial on the host response with simultaneous introduction of a biological component and the interconnections between the reactions elicited by the biomaterial and the biological components are yet to be fully characterized. The inflammatory response elicited by the presence of the biomaterial component may augment the immune response against the biological component through an adjuvant effect, and lead to unanticipated aggravation of the immune response. Especially as some biomaterials are explored for application in situations where high or low biomaterial adjuvant effect is desired, the understanding of the biomaterial adjuvant effect and its mechanisms are central to the effective design and selection of biomaterials for specific applications.

Poly(lactic-*co*-glycolic acid) (PLGA) is one such material, widely studied for many applications, in which its adjuvant effect may or may not be desired. Poly(lactic-*co*-glycolic acid) is a synthetic polymer composed of variable molar ratios of lactic and glycolic acids. Because its degradation can be controlled through polymer composition and its degradation products are biocompatible, PLGA has been extensively investigated (Hutchinson and Furr, 1986; Eldridge et al., 1991; Athanasiou et al., 1996; Mikos and Temenoff, 2000). The adjuvant effect of PLGA, as measured by antibody responses against antigens delivered with PLGA, has been evaluated as at least comparable if not greater than that elicited by known adjuvants such as alum and Complete Freund's Adjuvant (Ertl et al., 1996; Walker et al., 1998; Raghuvanshi et al., 2001).

Adjuvants produce their immunostimulatory effect through antigen presenting cells (APCs) such as the dendritic cells (DCs) (Singh and O'Hagan, 1999). Particulate adjuvants, including polymeric microparticles, are internalized by APCs, thereby activating them, inducing an immune response, or by creating a depot of antigen at the site of injection to prolong exposure (Hunter, 2002). In addition, adjuvants, especially those of bacterial origin, can enhance the immune response by activating APCs resulting in higher levels of expression of MHC and co-stimulatory molecules or by affecting cytokine release (Singh and O'Hagan, 1999). Interestingly, PLGA has been shown to act as an adjuvant through the induction of DC maturation (Jilek et al., 2004; Yoshida and Babensee, 2004).

Dendritic cells are professional APCs that bridge innate immunity, including inflammation, and adaptive immunity (Banchereau and Steinman, 1998; Banchereau et al., 2000). Upon encountering pathogen-associated molecular patterns, inflammatory

cytokines or other "danger signals", DCs undergo maturation. The state of maturation is characterized by loss of phagocytic and endocytic receptors, and downregulated pinocytosis, and upregulation of MHC and co-stimulatory molecules such as CD40, CD80, and CD86 (Sallusto et al., 1995; Winzler et al., 1997; Banchereau and Steinman, 1998; Banchereau et al., 2000). Maturing DCs migrate to lymphoid organs where they activate T cells for the initiation of the adaptive immune response (Banchereau and Steinman, 1998; Banchereau et al., 2000), and in this way bridge innate and adaptive immunity.

Many of stimulators of DC maturation, including adjuvants, activate DCs via a family of receptors known as the Toll-like receptors (TLRs). In particular, TLR2 and TLR4 have been implicated in the activation of DCs by endogenous and exogenous adjuvants. Poly-mannuronic acid, a component of a natural biomaterial, alginate, has been shown to activate DCs via TLRs 2 and 4 (Flo et al., 2002). Endogenous cell- or tissue-derived adjuvants such as heat shock proteins (Basu et al., 2000; Bethke, 2002) and degraded proteins that only appear upon tissue injury such as oligosaccharides of hyaluronan also activate DCs via TLR 4 (Termeer et al., 2000; Johnson et al., 2002; Termeer et al., 2002). As we have previously observed that PLGA activates human DCs as measured by increase in co-stimulatory and MHC molecule expression, inflammatory cytokine release, and allostimulatory capacity, this study sought to investigate the receptors involved in PLGA-induced maturation of DCs by focusing on TLRs. Because introduction of biomaterials into environment where proteins are present, both *in vivo* and *in vitro*, leads to instantaneous protein adsorption to material surface, PLGA-induced DC maturation is likely mediated by such adsorbed proteins. While studies demonstrating

PLGA-induced DC maturation were performed *in vitro*, the activation of DCs by PLGA *in vivo* is thought to be mediated by endogenous proteins. As such, we focused on the investigation of the roles of TLR2 and TLR4 on PLGA-induced maturation through receptor blocking studies with human DCs and using DCs from murine models of TLR4 deficient signaling.

METHODS:

Poly(lactic-co-glycolic acid) microparticle and film preparation

Poly(lactic-*co*-glycolic acid) microparticles (MPs) were prepared by a single emulsion solvent evaporation technique adapted from a previously described method (Wake et al., 1998; Yoshida and Babensee, 2004). Briefly, 500 mg of PLGA (molar ratio: 75:25, inherent viscosity: 0.69 dL/g in trichloromethane) (Birmingham Polymers, Birmingham, AL) was dissolved in 20 ml dichloromethane (DCM) (Sigma, St. Louis, MO) overnight at room temperature. On the second day, the PLGA-DCM solution was added to 200 ml 0.3% v/v aqueous poly(vinyl alcohol) (PVA) (Sigma), and homogenized for 2 min at 9000 rpm. Immediately after homogenization, 200 ml 2% (v/v) aqueous isopropanol (Sigma) was added to initiate precipitation, and the mixture stirred overnight at room temperature to evaporate the solvent. Resulting MPs were collected by centrifugation at 1000 rpm for 10 minutes, washed twice in 2% isopropanol, and three times in distilled deionized H_2O (ddH_2O). Microparticles were resuspended in ddH_2O and their size distribution characterized using a Coulter Multisizer II (Coulter Corporation, Miami, FL). Prior to use, the MP suspension was UV sterilized for 1 hr, and stored at 4°C for no longer than 3 days. Endotoxin content of PLGA MPs was

determined to below detection limit of the assay used (QCL-1000, Cambrex, Walkersville, MD).

Poly(lactic-*co*-glycolic acid) films were fabricated using a previously described casting technique without a porogen (Ishaug et al., 1997; Yoshida and Babensee, 2004). Briefly, 10% w/v 75:25 PLGA was dissolved in DCM overnight at room temperature. Solution of PLGA-DCM was poured onto a 50 mm Teflon Petri dish (Cole-Parmer, Vernon Hills, Illinois). Upon evaporation of the solvent and drying, the film was punched into an appropriate size, and washed for 1 hr in ddH_2O, changing water every 15 min. The film was dried 30 min per side in a sterile laminar flow hood, and UV sterilized for 30 min per side prior to use. For culturing with cells, the film was secured to the bottom of the tissue culture plate with a segment of sterile silicone tubing (Cole-Parmer). Endotoxin level of 4.5 mm diameter film was below limits of detection by QCL-1000 endotoxin assay (Cambrex).

Human dendritic cell culture

Human blood was obtained from volunteers with informed consent, according to a protocol approved by the Institutional Review Board of Georgia Institute of Technology, #00A018. Dendritic cells were derived from human peripheral blood mononuclear cells (PBMCs) using a previously described method (Romani et al., 1996) with some modifications, as previously reported (Yoshida and Babensee, 2004). Briefly, PBMCs were isolated from blood by differential centrifugation using Histopaque (Sigma). Upon lysing erythrocytes and washing, PBMCs were resuspended at a concentration of $5x10^6$ cells/ ml in RPMI-1640 containing 25 mM HEPES and L-glutamine (Gibco BRL, Grand

Island, NY) with 100 U/ ml penicillin-streptomycin (Cellgro, Herndon, VA) and 10% (v/v) heat inactivated filter sterilized fetal bovine serum (Invitrogen, Carlsbad, CA) (DC media). Cells were plated in a volume of 10 ml/ plate in a 100 x 20 mm tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and incubated for 2 hrs to select for adherent monocytes. After the incubation, plates were washed 3 times with warm DC media to remove non-adherent cells, and the remaining adherent cells were supplied with 10 ml DC media and incubated with 2000 U/ ml GM-CSF and 1600 U/ ml IL-4 (Peprotech, Rocky Hill, NJ). On day 5 of culture, loosely- and non-adherent cells containing DCs were collected by centrifugation for 10 min at 1100 rpm, and resuspended in DC media with 1000 U/ ml GM-CSF and 800 U/ ml IL-4 for use in various experiments.

In vitro *antibody blocking experiments*

The role of particular receptors, namely CD14, TLR2, and TLR4, in PLGA-induced human DC maturation was evaluated *in vitro* using blocking antibodies. Immature DCs collected on day 5 of culture were resuspended in DC media with 1000 U/ ml GM-CSF and 800 U/ ml IL-4 at a concentration of $1x10^6$ cells/ well in 48 well plate (Becton Dickinson). Dendritic cells were incubated with or without various blocking antibodies alone or in combination or with isotype control antibodies for 1 hr at room temperature. Following antibodies were used: $IgG_1\kappa$ isotype (clone 107.3, no sodium azide, low endotoxin, Becton Dickinson Pharmingen, San Jose, CA), $IgG_{2a}\kappa$ isotype (clone G155-178, no sodium azide, low endotoxin, Becton Dickinson Pharmingen), CD14 (clone MEM-18, azide-free, Cell Sciences, Canton, MA), TLR2 (clone TL2.1, no

sodium azide, Biocarta, San Diego, CA), or TLR4 (clone HTA125, no sodium azide, Biocarta). After blocking with the respective antibodies for 1 hr, DCs were treated with a maturation stimulus of 1 μg/ ml LPS (Sigma, St. Louis, MO), 5:1 MP: cell ratio of PLGA MPs, PLGA film, or left untreated for additional 24 hrs.

Human Tumor Necrosis Factor α (TNFα) ELISA

Cell culture supernatant from human DC culture was collected at 5 hrs after the initial treatment with the maturation stimulants. The supernatant was cleared and stored at -20°C until analysis. The amount of TNFα produced by DCs in the cell culture supernatant was analyzed by ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's directions.

Murine bone marrow-derived dendritic cell culture

Bone marrow-derived DCs were prepared from mice in accordance with protocol approved by Emory University IACUC (protocol number 034-2003), based on previously published methods with some modifications (Inaba et al., 1992). Male, 5-7 week old C57BL6 (haplotype I-A^b), C3H/HeJ (haplotype I-A^k) and C3H/HeOuJ (haplotype I-A^k) were purchased from Jackson Laboratories (Bar Harbor, ME). C3H/HeJ animals have a mutation in toll-like receptor 4 gene, $Tlr4^{Lps\text{-}d}$, resulting in LPS hyporesponsivity, and have been extensively used as a model for TLR4 signaling (Ohashi et al., 2000; Takeuchi et al., 2000; Termeer et al., 2002). Of the several corresponding wild type substrains of C3H, C3H/HeOuJ was selected, as both strains of mice were available from the same

commercial source. For each strain, 6 animals were used for each experiment. Femur and tibia from mice were dissected, and muscle and tissue removed. Cleaned bones were placed in 70% ethanol in 60 mm suspension dish for 1 min, washed twice in PBS, and placed into culture media composed of DMEM (Cellgro, Herndon, VA) with 1% penicillin-streptomycin (Cellgro), 1% non-essential amino acids (Cellgro), 1% sodium pyruvate (Cellgro), 1% HEPES (Cellgro), 0.1% 2-mercaptoethanol (Invitrogen), with 10% heat inactivated fetal bovine serum (Invitrogen). Ends of the bones were cut, and bone marrow flushed with at least 5 ml of media using a 26G needle. The bone marrow suspension was strained using a 70 μm cell strainer (Becton Dickinson), cells collected by centrifugation at 1500 rpm for 5 min, and erythrocytes lysed with ammonium chloride (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Bone marrow cell suspension was then washed twice in PBS with 10% FBS, resuspended at $1.5x10^6$/ ml with 20 ng/ ml GM-CSF (Sigma) and 20 ng/ ml IL-4 (Sigma), and plate 3 ml/ well into 6 well plates (Corning), and incubated at 37°C with 95% relative humidity and 5% CO_2. On day 3, cells were fed by exchanging half of the media with fresh media containing 20 ng/ ml GM-CSF and 20 ng/ ml IL-4. On day 6 of culture, loosely- and non-adherent cells were collected, and washed twice in PBS with 2% FBS and 2 mM EDTA, and processed for CD11c+ magnetic separation per manufacturer's directions (Miltenyi Biotech, Auburn, CA).

Cells positive for CD11c were considered as iDCs, and used for subsequent experiments. These CD11c+ iDCs were resuspended at $1x10^6$ cells/ ml, and plated at 1 ml/ well in 24 well plate. Effect of PLGA MPs or film on DC maturation was tested by treating the cells with 5:1 MP: cell ratio of PLGA MPs or with PLGA film placed at the

bottom of the well for 24 hrs. For negative and positive controls, iDCs were left untreated or treated with 1 μg/ ml LPS, respectively for 24 hrs.

Murine Tumor Necrosis Factor α (TNFα) and IL-6 ELISA

Cell culture supernatant from murine DC culture was collected at 4 hrs after the initial treatment with the maturation stimulants. The supernatant was cleared and stored at -20°C until analysis. The amount of TNFα and IL-6 produced by DCs in the cell culture supernatant was analyzed by ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's directions.

Cell surface marker expression

Extent of DC maturation was assessed by measuring the expression of cell surface markers by flow cytometry. Dendritic cells were harvested, and resuspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM glucose, 30 mM HEPES) containing 1% (v/v) human serum albumin (HSA) and 1.5 mM $CaCl_2$. For human DCs, cells were stained with mouse anti-human monoclonal antibody against CD40 (clone B-B20; $IgG_1\kappa$), CD80 (clone BB1; IgMκ), CD86 (clone BU63; $IgG_1\kappa$) (Southern Biotech, Birmingham, AL), CD83 (clone HB15a; IgG_2b) (IO Test Immunotech Beckman Coulter, Marseille, France), HLA-DQ (clone TU169; $IgG_{2a}\kappa$), or HLA-DR (clone TU36; $IgG_{2a}\kappa$) (Becton Dickinson Pharmingen, San Diego, CA) for 1 hr at 4°C. For murine DCs, cells were stained with antibodies against CD86 (clone GL1; $IgG_{2a}\kappa$), CD80 (clone 16-10A1, $IgG_2\kappa$), CD11c (clone HL3, $IgG_1\lambda2$), $I\text{-}A^b$ (clone AF6-120.1, $IgG_{2a}\kappa$), or $I\text{-}A^k$ (clone 10-3.6, $IgG_{2a}\kappa$) (Becton Dickinson Pharmingen) for 1 hr at 4°C.

All cells were analyzed using BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA) and BD FACS DiVa Option 2.0 (Becton Dickinson Biosciences, San Jose, CA).

Statistical Analysis

Statistical analysis was performed using general linear model ANOVA with Minitab software (Version 13.20, Minitab Inc., State College, PA), and pairwise comparison to appropriate control group as indicated was made, unless otherwise indicated, with *p*-values of less than or equal to 0.05 considered to be significant. Power analysis for sample size of animal studies was confirmed using 2-sample t test, with a sigma value of 0.8 using Minitab.

RESULTS:

Pretreatment of human immature dendritic cells with TLR2 blocking antibody enhances maturation

To investigate the receptors involved in PLGA-induced DC maturation, *in vitro* receptor blocking experiments were performed. Receptors known to be involved in DC signaling and phagocytosis were evaluated to assess the mechanism of maturation as well as receptors that mediate PLGA MP uptake. In particular, CD14, CD36, CD51, DC-SIGN, mannose receptor, TLR2, and TLR4 were evaluated. All of these receptors were present on DCs, as measured by flow cytometry (Figure A4, APPENDIX). Blocking of receptors using antibodies or known ligands to these receptors within the range tested was not able to block the LPS- or PLGA MP-induced maturation (Figure A5, APPENDIX). Likewise, blocking receptors by pretreating DCs with TLR2, TLR4, or CD14 antibody alone or in combination was unable to reduce LPS- or PLGA-induced DC maturation as measured by CD83 and CD86 expression (Figure 8-1). However, blocking iDC receptors using TLR2 antibody alone resulted in enhanced expression of CD83 and CD86 without any additional maturation stimulus (Figure 8-1). Similarly, pretreatment of DCs with these antibodies also increased the fold increase in the expression of these markers (Figure 8-1b). Because many of the DC agonists exert their maturation effect by signaling through TLR4 alone or in combination with other receptors such as CD14 and TLR2, the effect of simultaneously blocking these receptors in combination was examined. Blocking of receptors in combination did not greatly affect the LPS- or PLGA-induced increase in marker expression. However, with antibody combinations in which anti-TLR2 or anti-CD14 antibody was present, iDCs expressed higher than

expected levels of CD83 and/ or CD86 as observed with blocking receptor with TLR2 antibody alone (Figure 8-1a, b).

Similar to the observation with maturation marker expression, pretreatment of iDCs with TLR2 antibody and combinations thereof, without additional maturation stimulus resulted in increased release of TNFα (Figure 8-2). The increase in TNFα released with the addition of LPS as maturation stimulants was not significantly altered by the pretreatment of DCs with any of the blocking antibodies alone or in combination. PLGA MP-induced increase in TNFα release was further enhanced by the pretreatment of DCs with TLR2 blocking antibody alone or in combination with other antibodies.

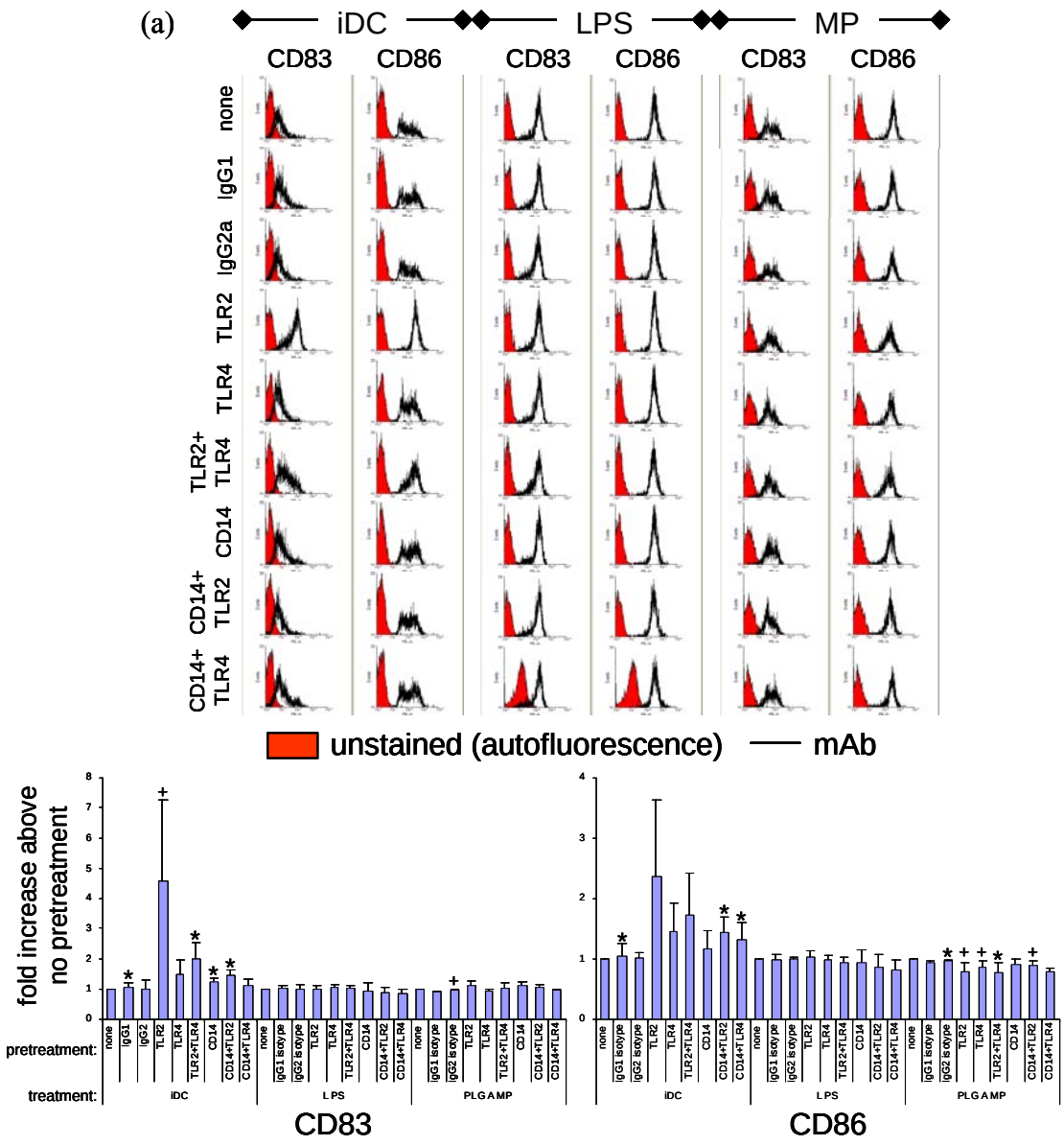

Figure 8-1: Pretreatment of iDCs with TLR2 blocking antibody increases marker expression.

Immature DCs were pretreated with isotype control antibody or antibodies against respective receptors for 1 hr at room temperature, and left untreated or treated with LPS or PLGA MPs for additional 24 hrs. LPS- and PLGA MP- treated DCs increased their expression of CD83 and CD86. Pretreatment of iDCs with TLR2 antibody alone or in combination increased the expression of CD83 and CD86 above those expressed by isotype control antibody-pretreated cells. This experiment was repeated three times with similar results, and representative results are shown (a). Fold increase in gMFI values for each pretreatment groups as compared to that of no antibody pretreatment control (none) revealed statistical significance of the findings (b). mean±SD, n=3. */+: $p<0.05$/ 0.1, respectively, compared to none.

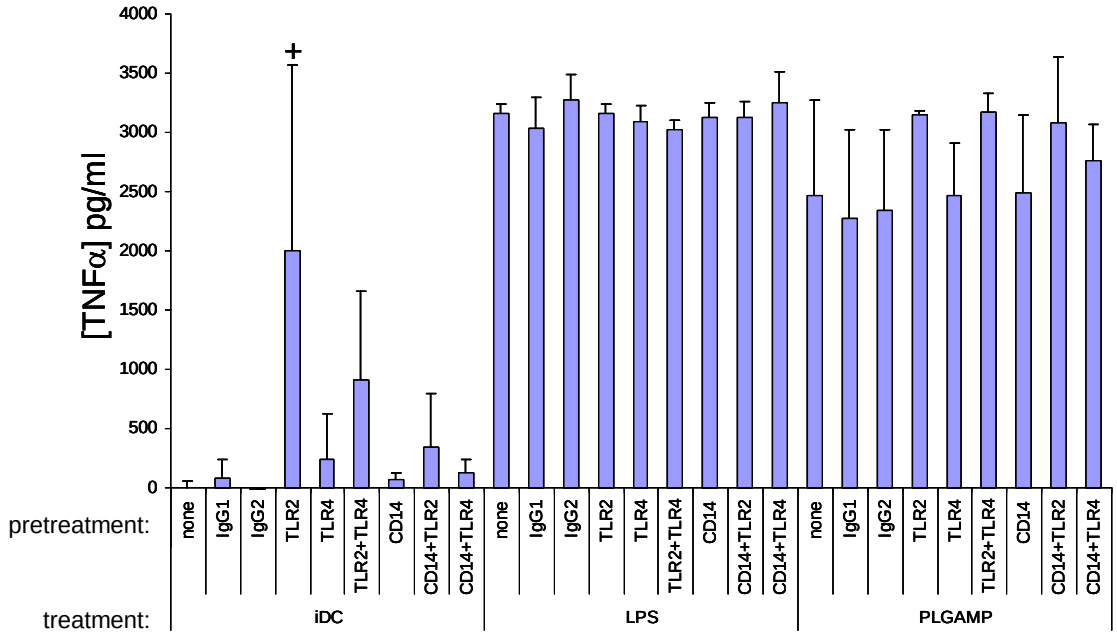

Figure 8-2: Pretreatment of iDCs with TLR2 blocking antibody increases TNFα release.

Immature DCs were pretreated with isotype control antibody or antibodies against respective receptors for 1 hr at room temperature, and left untreated or treated with LPS or PLGA MPs for additional 24 hrs. LPS- and PLGA MP- treated DCs secreted increased amount of TNFα in comparison to iDCs. Increase in TNFα production induced by LPS was not blocked by pretreatment of DCs with the blocking antibodies. Production of TNFa by iDCs was enhanced by addition of TLR antibodies. mean±SD, n=3. +: $p<0.1$ compared to none.

Bone marrow-derived DCs from C57BL6 mice increase CD86 expression and cytokine secretion

As results from *in vitro* blocking studies of human DCs were conflicting, with increased DC maturation due to pretreatment of antibodies alone without maturation stimulants and the lack of blocking effect on LPS-induced maturation, another approach was taken to further investigate the role of CD14, TLR2, and TLR4 in the PLGA-induced DC maturation. Commercially available murine model of LPS-dependent signaling, C3H/HeJ strain was chosen for this purpose. Prior to using DCs derived from C3H/HeJ mice, ability of PLGA to induce DC maturation in wild type C57BL6 strain was evaluated. Morphology of bone marrow-derived DCs from C57BL6 mice treated with PLGA MPs or film were similar to that of LPS-matured DCs with dendritic processes (Figure 8-3).

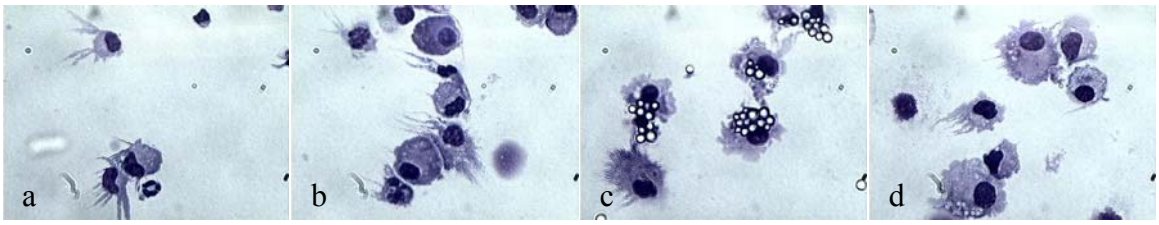

Figure 8-3: Bone marrow-derived DCs from C57BL6 mice treated with PLGA MPs or film possess morphology similar to DCs matured with LPS.
Bone marrow-derived iDCs from C57BL6 mice were left untreated (a) or treated with LPS (b), PLGA MPs (c), or PLGA film (d) for 24 hrs. Dendritic cells treated with PLGA MPs or films show dendrites as do LPS-matured DCs and iDCs to a lesser extent. This experiment was repeated six times with similar results, and representative results are shown.

Expression levels of co-stimulatory molecules CD80 and CD86, as well as DC marker CD11c and murine MHC molecule I-A^b were measured. Expectedly, expression levels of CD11c and I-A^b were not greatly altered by the treatment of DCs with LPS or PLGA MPs or films (Figure 8-4a). Bone marrow-derived DCs from C57BL6 animals increased expression of CD80 and CD86 in response to treatment with LPS, PLGA MP, or PLGA film. However, changes in fold increase were not readily observed with the different treatment groups (Figure 8-4b).

As one important consequence of activation of DCs via TLRs is the production of pro-inflammatory cytokines (Akira et al., 2001), cell culture supernatants from DCs treated with or without LPS, PLGA MPs or PLGA film were analyzed for levels IL-6 and TNFα using ELISA. As expected, LPS-matured DCs secreted high amounts of TNFα and IL-6 in comparison to iDCs (Figure 8-5a, b). In addition, PLGA MP- and PLGA film-treated DCs also released substantially higher amounts of TNFα and IL-6 above those secreted by iDCs (Figure 8-6). Taken together, these results suggest that treatment with PLGA MPs or PLGA film induce maturation of bone marrow-derived DCs from C57BL6 mice.

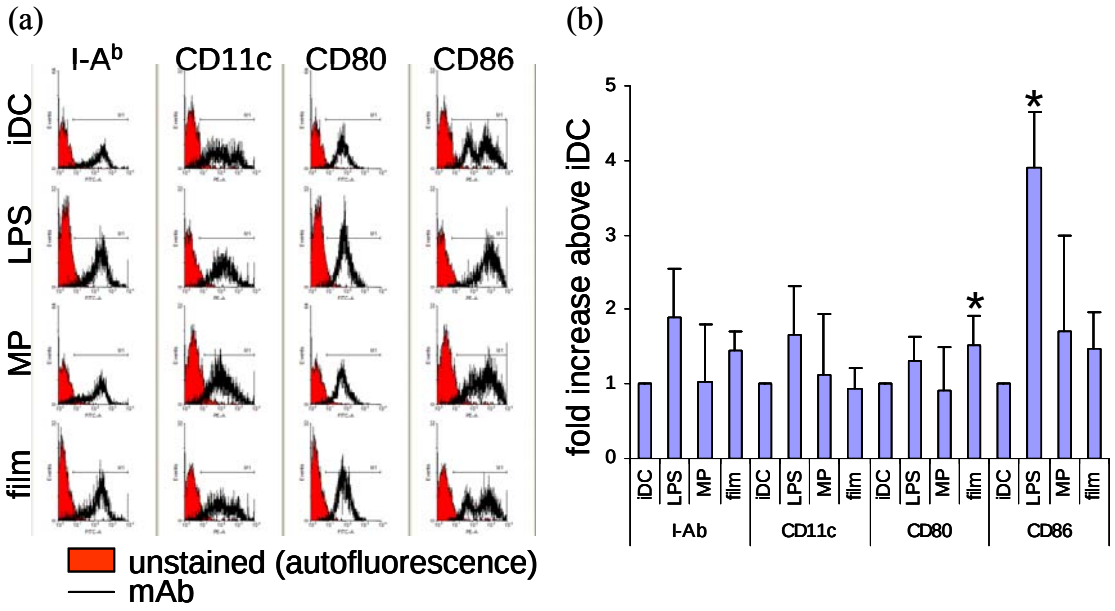

Figure 8-4: PLGA induces increased expression of CD86 on bone marrow-derived DCs from C57BL6 mice.
Bone marrow-derived iDCs from C57BL6 mice were left untreated or treated with LPS, PLGA MPs, or PLGA film for 24 hrs. Expression of DC markers were measured by flow cytometry. Treatment of iDCs with PLGA MPs enhanced expression of CD86, whereas the expression of other markers was not noticeably changed. This experiment was repeated six times with similar results, and representative results are shown (a). Fold increase in gMFI values for each treatment groups as compared to that of iDC control revealed statistical significance of the findings (b). mean±SD, n=6. *: $p<0.05$ compared to iDC.

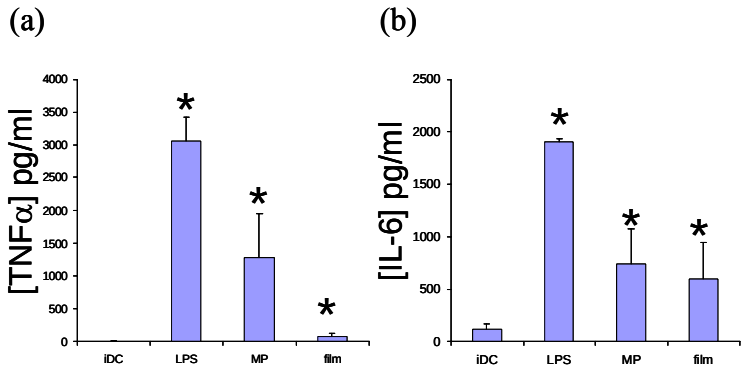

Figure 8-5: PLGA induces increased secretion of TNFα and IL-6 by bone marrow-derived DCs from C57BL6 mice.
Bone marrow-derived iDCs from C57BL6 mice were either left untreated or treated with LPS, PLGA MPs, or with PLGA film for 24h. Cell culture supernatant at 4 hrs after the initial treatment of DCs was assessed for TNFα (a) or IL-6 (b) content. Dendritic cells treated with LPS secreted high amount of both cytokines. As compared to iDCs, PLGA MP-treated DCs secreted higher amounts of both TNFα and IL-6, whereas PLGA film induced higher secretion of IL-6 but not TNFα. mean±SD, n=6. *: $p<0.01$ compared to iDCs.

Bone marrow-derived DCs from C3H/HeOuJ or C3H/HeJ mice do not mature in response to treatment with PLGA MPs or film

As enhanced maturation in response to treatment with PLGA was observed with murine DCs derived from the bone marrow of C57BL6 mice, role of TLR4 in particular was further investigated. For this purpose, bone marrow-derived DCs from commercially available murine model, C3H/HeJ strain, were used. C3H/HeJ mice have a mutation in toll-like receptor 4 gene, $Tlr4^{Lps\text{-}d}$, are LPS hyporesponsive, and have been used as a model for investigating the involvement of TLR4 in signaling (Ohashi et al., 2000; Takeuchi et al., 2000; Termeer et al., 2002). Several corresponding wild type substrains of C3H are used in parallel to studies of C3H/HeJ mice, but for this application, C3H/HeOuJ was selected, as both strains of mice were available from the same commercial source. Treatment of DCs from C3H/HeJ or C3H/HeOuJ mice with LPS, PLGA MPs, or PLGA film did not result in any difference in cell morphology (Figure 8-6).

Expression of maturation markers surveyed as compared to iDCs was not changed in DCs from C3H/HeJ nor C3H/HeOuJ mice upon PLGA treatment (Figure 8-7a, b). Similarly, changes in fold increase in the expression of these markers were not observed to be significant, except for a slight increase in I-Ak and CD86 expression in response to LPS for DCs derived from C3H/HeJ animals (Figure 8-7c, d).

To further evaluate the role of TLR4 in PLGA-induced murine DC maturation, secretion of pro-inflammatory cytokines TNFα and IL-6 was measured. Dendritic cells derived from C3H/HeOuJ animals treated with LPS increased secretion of both TNFα and IL-6 as expected (Figures 8-8c ,d). In particular, the secretion of IL-6 was at a level

comparable to that secreted by DCs derived from C57BL6 mice. While at a statistically significantly lower extent ($p<0.01$ for both TNFα and IL-6), DCs derived from C3H/HeJ animals also secreted TNFα and IL-6 in response to LPS (Figures 8-8a, b). Dendritic cells from neither strain responded to treatment with PLGA MPs, but did so to a very low extent in response to PLGA film (Figure 8-8a, d), in agreement with results from marker expression as summarized above.

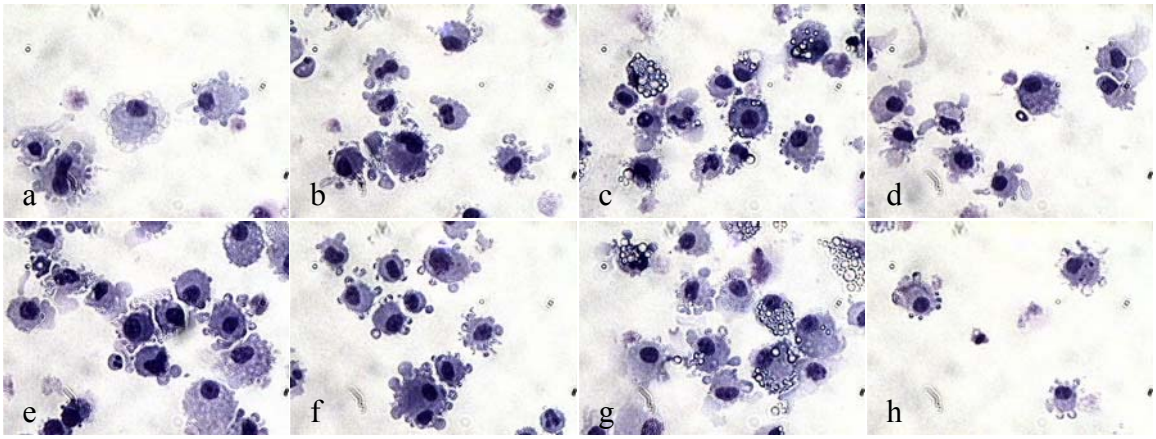

Figure 8-6: Bone marrow-derived DCs from C3H/HeJ (a-d) and C3H/HeOuJ (e-h) mice treated with PLGA MPs or film possess morphology similar to DCs matured with LPS.
Bone marrow-derived iDCs from C3H/HeJ or C3H/HeOuJ mice were left untreated (a,e) or treated with LPS (b,f), PLGA MPs (c,g), or PLGA film (d,h) for 24 hrs. Dendritic cells treated with PLGA MPs or films show veils as do LPS-matured DCs and iDCs to a lesser extent. This experiment was repeated six times with similar results, and representative results are shown.

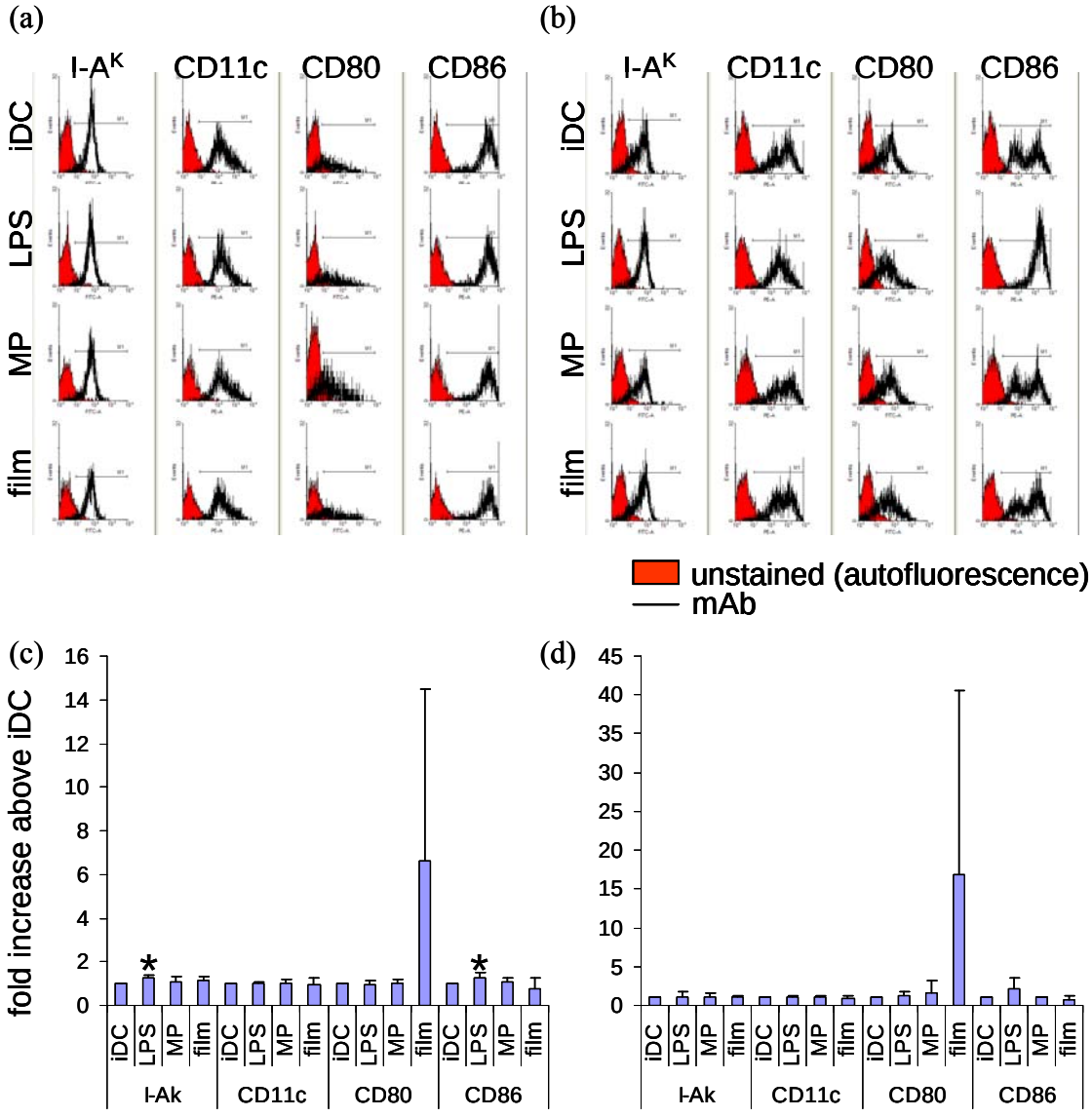

Figure 8-7: PLGA does not affect expression of DC markers on bone marrow-derived DCs from C3H/HeJ or C3H/HeOuJ mice.
Bone marrow-derived iDCs from C3H/HeJ (a, c) or C3H/HeOuJ (b, d) mice were either left untreated or treated with LPS, PLGA MPs, or with PLGA film for 24 hrs. Expression of DC markers were measured by flow cytometry (a, b). Treatment of C3H/HeJ DCs with any of the maturation stimulants, LPS, PLGA MPs, or PLGA film did not significantly alter the expression of the DC markers measured. This experiment was repeated 6 times with similar results, and representative results are shown (a, b). Fold increase in gMFI values for each treatment groups as compared to that of iDC control revealed statistical significance of the findings (c, d). mean±SD, n=6. *: $p<0.05$ compared to iDC.

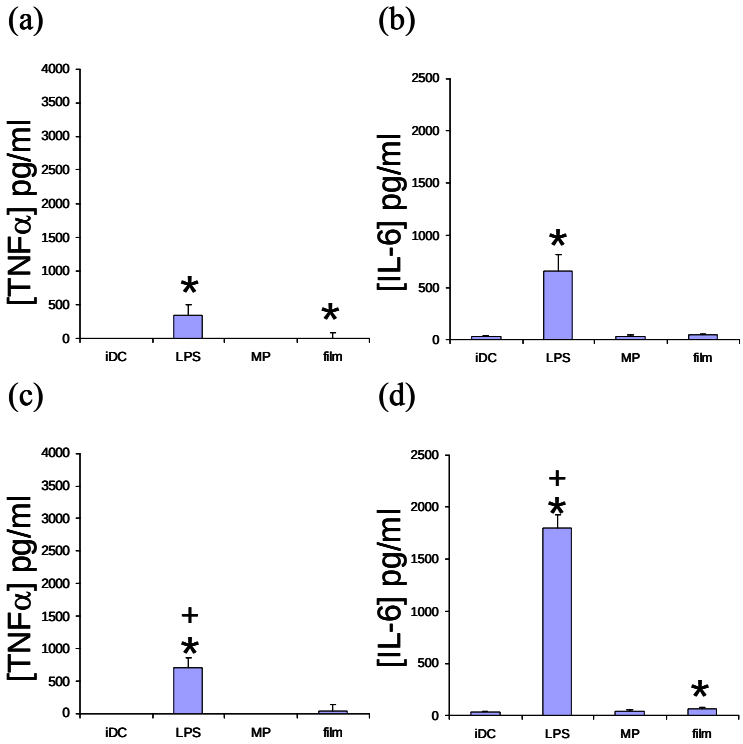

Figure 8-8: PLGA does not greatly alter secretion of TNFα and IL-6 by bone marrow-derived DCs from C3H/HeJ or C3H/HeOuJ mice.
Bone marrow-derived iDCs from C3H/HeJ (a, b) or C3H/HeOuJ (c, d) mice were either left untreated or treated with LPS, PLGA MPs, or with PLGA film for 24 hrs. Cell culture supernatant at 4 hrs after the initial treatment of DCs was assessed for TNFα (a, c) or IL-6 (b, d) content. However, DCs from C3H/HeOuJ animals consistently secreted higher amounts of both TNFα (c) and IL-6 (d) as compared to cells from C3H/HeJ animals (a, b). mean±SD, n=6. *: $p<0.01$ compared to iDCs for each respective graph. +: $p<0.01$ compared to LPS treatment group of DCs derived from C3H/HeJ animals. TNFα and IL-6 secretion by LPS-treated DCs were significantly higher by DCs derived from C3H/HeOuJ mice as compared to those derived from C3H/HeJ mice ($p<0.01$).

DISCUSSION:

Poly(lactic-*co*-glycolic acid) is widely explored for various applications in which its adjuvant effect may or may not be desirable. As PLGA has been previously shown to induce DC maturation (Jilek et al., 2004; Yoshida and Babensee, 2004), the aim of this study was to investigate the receptors that may be involved in this PLGA-induced DC maturation. Within the concentrations tested, none of the antibodies or ligands used to block receptors of human DCs revealed clear indication of the role of particular receptors in PLGA-induced DC maturation. Moreover, blocking of human iDC receptors with some of the TLR antibodies resulted in increased expression of maturation markers and cytokine release, suggesting that the pretreatment by antibody alone activated the DCs (Figures 8-1, 8-2). This activation of human DCs in presence of antibodies may be attributed to a few factors. First is that although unlikely, there may have been contaminating endotoxin present in the antibody preparation. A second speculation is that blocking antibodies may have resulted in crosslinking of the Fc portion of immunoglobulin (Ig) G receptor (FcγR) on DCs, resulting in their activation. Such activation of leukocytes by antibody crosslinking has been observed with DCs (Regnault et al., 1999; Banki et al., 2003) as well as with neutrophils (Simon et al., 1995) and monocytes (Drechsler et al., 2002). In particular, crosslinking of CD32, or FcγRII, using immobilized IgG has been shown to induce maturation of human monocyte-derived DCs via activation of NFκB pathway (Banki et al., 2003). Dendritic cells matured by this crosslinking of FcγRII were functionally mature, as indicated by their ability to induce allogeneic T cell proliferation and cytokine production, which could be inhibited by the addition of FcγRII-blocking monoclonal antibody. In accord with other studies (Sallusto

and Lanzavecchia, 1994; Banki et al., 2003), this particular Fcγ receptor, CD32, has been confirmed to be present on our DC preparation by flow cytometry (Figure A4, APPENDIX). The binding of anti-TLR antibody to DCs and subsequent crosslinking of Fc portions via DC-bound immunoglobulin may have led to the unexpectedly high expression of CD86 and TNFα production by DCs upon pretreatment with this antibody. In another study, polymeric microspheres with covalently attached IgG on the surface, similar to cell-bound IgG, has been shown to increase CD83 expression (Kempf et al., 2003), supporting the hypothesis of DC maturation due to cell-bound TLR2 antibody in our study. Although blockade of FcγR may obstruct the enhanced DC maturation effects observed, a recent study has demonstrated that blocking of inhibitory FcγRII, specifically FcγRIIb, induces human DC maturation as measured by increased co-stimulatory molecule expression and IL-12p70 secretion (Dhodapkar et al., 2005). As immune complexes and cell-bound immunoglobulins present in normal human serum may be enough stimulus to induce DC maturation via FcγR (Regnault et al., 1999), such studies underscore the complexities of different FcγRs and other factors involved in DC maturation. Another possibility to obviate the blocking antibody-induced DC maturation may be to prepare Fab fragments to avoid crosslinking of Fc portion of the IgG.

As expected, LPS-induced production of IL-6 and TNFα by DCs from C3H/HeOuJ mice was consistently higher than those produced by DCs from C3H/HeJ mice. However, while at a lower level, DCs from C3H/HeJ mice did secrete measurable amounts of TNFα and IL-6 in response to LPS. Similarly, secretion of low amounts of LPS-induced MIP-2, but not complete abrogation, by cells from C3H/HeJ strains as compared to the control C3H/HeN strain has been observed (Nikulina et al., 2004). This

response to LPS in cytokine production may have been due to unintentional favorable environmental conditions for induction of DC maturation, resulting in generally elevated state of DC maturation. This may be likely since while cytokine secretion by iDCs from C3H/HeJ mice was low, CD86, whose expression is upregulated on maturation, was already fairly highly expressed by iDCs. Another potential cause for the secretion of cytokines in response to LPS in this strain may be due to the presence of impurities in the LPS preparation used in this study. Presence of DC maturation agonists that act via receptors other than TLR4 may have caused the production of TNFα and IL-6 without greatly affecting co-stimulatory marker expression. Use of ultra-pure grades of LPS, as well as confirming the LPS-hyporesponsiveness of this strain using cells other than DCs, such as T cell proliferative capacity in response to LPS, may help address this issue, and validate utilization of C3H/HeJ strain to further investigate the role of TLR4.

While PLGA was capable of inducing maturation of bone marrow-derived DCs of C57BL6 mice, PLGA MPs or film were unable to induce maturation of DCs from C3H/HeOuJ mice. This result is peculiar, given that LPS-induced signaling in DCs from C3H/HeOuJ mice was intact, as supported by increased expression of co-stimulatory molecules and TNFα and IL-6 production in response to LPS. Moreover, while LPS-induced production of IL-6 by DCs from C3H/HeOuJ mice was comparable to that produced by DCs from C57BL6 mice, production of TNFα was substantially lower. This difference in cytokine production in response to LPS may be attributed to the difference in MHC haplotypes of the 2 strains of mice. Haplotypes of MHC have been shown to differentially regulate production of cytokines (Dieli et al., 1995; Caruso et al., 1996) as well as T cell recognition of bacterial antigens (Pichugin et al., 1998; Kamath et al.,

2004). Specifically, production of TNFα by T cells from bacteria-infected animals upon *in vitro* restimulation with bacterial antigens has been shown to be significantly lower by cells from BALB/c (H-2^k) as compared to those secreted by T cells from MHC congenic BALB.B10 (H-2^b) or C57BL6 (H-2^b) (Huygen et al., 1992).

Because cytokine secretion may be under regulation of MHC complexes, comparison between C57BL6 and C3H substrains may be complicated by the differing haplotypes of these two strains. Even within the C3H strains, the heterogeneity in the substrains makes the selection of appropriate control strain for the HeJ substrain difficult; significant heterogeneity in morbidity to *M. tuberculosis* infection amongst four substrains of C3H including HeJ and HeOuJ (Kamath et al., 2003), as well as differing susceptibility to autoimmune diseases (Glant et al., 2001) have been documented. As we have tried to control for as many environmental factors as possible, by housing the animals in the same facility under the same conditions, handling the mice in the same manner, obtaining all strains from the same vendor, the factors that may have caused this unforeseen difference in results obtained from C57BL6 and C3H animals are likely genetic. With these differences regulated by MHC haplotypes, it may be worthwhile to investigate the effect of PLGA on murine DC maturation using other strains of mice as controls. Furthermore, because the differences observed were in the production of cytokines, investigation of polarization of T cells by cytokines would also be of interest. Ultimately, the relative effect observed with cells from C3H/HeJ in response to any stimulants, in this case effect of PLGA contact on DC maturation, will depend in part on the choice of the control strain (Kamath et al., 2003).

As such, repeating these experiments using DCs from other C3H substrain(s) of wild type controls may be necessary to provide conclusive results to either delineate or rule out the role of TLR4 in PLGA-induced DC maturation. In addition, experiments using DCs derived from transgenic animal models of MyD88 or TLR2 deficiency would be of interest. Originally, our intent was to investigate the role of MyD88 first, as this adaptor protein associates with TLR2 and TLR4 and is implicated in the intracellular signaling mediated by these 2 TLRs (Kaisho and Akira, 2001). However, logistical inaccessibility to this particular animal model has prompted us to study the role of TLR4 using commercially available C3H/HeJ strain. Because there exist MyD88-dependent and independent pathways of DC activation via TLRs (Kaisho and Akira, 2001), lack of response by DCs from MyD88-deficient murine models would not necessarily rule out the role of TLR2 and TLR4. To specifically study the contributions of particular TLRs in mediating biomaterial-induced DC maturation, one may need to use a multivariate approach of *in vitro* and *in vivo* methods, using model cell lines lacking particular receptors and cells from animal models with deficiencies in particular receptors. If results from additional investigations do not support the involvement of TLR2 or TLR4 in the PLGA-induced DC maturation, focused efforts on receptors known to mediate activation by proteins adsorbed on material surfaces such as FcγR, integrins, or phagocytic receptors that may play a role in the internalization of PLGA MPs may provide insight into PLGA-induced DC maturation.

CHAPTER 9

CONCLUSIONS AND FUTURE WORK

Characterization of biomaterials and their associated immune and inflammatory responses in the context appropriate for their intended use is of critical relevance to the development of novel biomaterials and modifications to currently existing biomaterials. This thesis research contributed to the field of biomaterial science in modern medicine by assessing and demonstrating differential adjuvant effects of two model biomaterials, agarose and PLGA, focusing on DC maturation. In addition, panel of assays that can be utilized for screening biomaterials to be used in multiple applications were developed. As DCs can initiate adaptive immune response upon maturation, evaluation of DC maturation *in vitro* provides a basis for the biomaterial adjuvant effect *in vivo* and the predisposition of a biomaterial to induce adaptive immunity to associated biological components, which may result in graft rejection.

Studies presented herein provide a framework for assessing biomaterial adjuvant effect and its associated mechanisms. Short-term, immediate experiments that can help address particular issues have been suggested throughout this thesis. However, to further develop biomaterial-screening protocols towards the development of biomaterial-centered immunomodulatory strategies via control of DC phenotype, as well as understand the mechanisms by which biomaterials are recognized by DCs to result in their maturation, several key areas may be investigated.

While investigations summarized in this thesis evaluated several DC phenotypes to exemplify maturation, a more in-depth characterization of this maturation as well as

the subsequent immune response elicited would be of benefit. For example, given the functionally different subsets of DCs and the differential response they elicit, characterization of DC subset induced by biomaterials would be important. Specifically, the types of cytokines released by DCs cultured in contact with biomaterials, as well as those released by T cells stimulated by the biomaterial-treated DCs may begin to elucidate the type of response that can be expected when such biomaterials are introduced *in vivo*. In this project, analysis of inflammatory cytokines secreted by DCs cultured with PLGA or with agarose was assessed, but only at one time point of 24 hrs after stimulation (Figure A3, APPENDIX). A time course of cytokine secretion, as well as mRNA levels of these cytokines as well as DNA microarray analyses of regulation at the genetic level, may reveal more differential effects of biomaterials. Using the cytokine profile and determining the ensuing immune response elicited via T cells, either through Th1/ Th2 polarization or regulatory mechanisms may provide means of inducing host acceptance and tolerance of tissue engineered grafts. In addition, the maturation state of DCs can also be evaluated through their ability for migration. Specifically, measurment of expression of CCR7 on DCs required for migration (Forster et al., 1993), as well as their propensity for migration towards CCR7 ligands such as CCL19 and CCL21 using a transwell system would be of importance in better understanding the effect of biomaterials on state of DC maturation.

Moreover, *in vivo* characterizations of biomaterial-induced DC maturation may offer insight into mechanisms of DC maturation. In our study, DTH was used as a gauge of *in vivo* induced DC maturation, and showed that OVA delivered with PLGA MPs induced comparable degree of response than did OVA delivered with known adjuvant

CFA (CHAPTER 5). Other aspects of biomaterial adjuvant effect to be evaluated may include introduction of biomaterial with a model antigen into a host, and measuring subsequent production of cytokines and proliferation of cells from the draining lymph nodes in response to re-stimulation with the model antigen, as well as assessing systemic antibody responses and isotypes as compared to appropriate controls. The antigen-specific proliferation of T cells would be indicative of CD4 T cell immunity. While this study attempted to assess the relative effectiveness of PLGA MPs to complete Freund's adjuvant, standard adjuvant used in experimental animal models, both *in vivo* (CHAPTER 5) and *in vitro* (APPENDIX), *in vitro* evaluation of adjuvant effect elicited by biomaterial MPs as compared to clinically used particulate adjuvant such as alum could provide insight into clinical relevance of PLGA MPs as vaccine adjuvant.

Although these additional characterizations may provide further evidence that may begin to elucidate the mechanisms of biomaterial-induced DC maturation, it would be of great interest to identify the molecules involved in the biomaterial-induced maturation. In this thesis project, the role of TLRs was examined *in vitro* using blocking antibodies and animal models deficient in the particular receptor of interest (CHAPTER 8). In our hands, the specific antibodies reported herein were unable to be fully utilized for blocking studies as is as their presence resulted in unintentional activation of the cells as measured by increased maturation marker expression and cytokine release. This activation was thought to be due to the crosslinking of Fc receptors, which may be alleviated by use of Fab fragments of antibodies, and titration to determine the appropriate concentration required for blocking the function of these receptors. Alternatively, as other groups have reported successful use of blocking antibodies against

these same receptors, a different source of antibody, likely not commercial, may need to be acquired (Akashi et al., 2000).

In this study, fragmentation of antibody was not pursued; rather, efforts were focused on the elucidation of the role of TLR4 using DCs from animal models of TLR4 deficiency. However this study was made complicated by high baseline activation of iDCs from C3H/HeJ animals as measured by maturation marker expression, and significantly lower extent of maturation response to LPS or PLGA by DCs from C3H/HeOuJ control animals as compared to DCs from C57BL6 animals as measured by marker expression and inflammatory cytokine release. To further pursue this approach in the determination of the role of TLR4 in PLGA-induced DC maturation, several experimental methods can be taken. Prior to using cells of C3H/HeJ strain, it may be of benefit to determine the baseline level of DC activation in response to LPS in the control strains. Cells from C3H/HeOuJ mice or other C3H substrains such as C3H/HeSnJ and C3HeB/FeJ which originated from the same stock as C3H/HeJ strain and carry the Lps^n allele (Qureshi et al., 1999) may be used. As mentioned in CHAPTER 8, as the C3H substrains have differing haplotypes than C57BL6 mice, comparison between these two different strains may not be appropriate. For this and subsequent application, the use of ultra-pure grades of LPS is vital given that TLR4 deficiency confers LPS hyporesponsiveness, and presence of any impurities present in the LPS preparation may act as agonists for other receptors and activate cells. In addition, waiting longer for the animals to quiesce in the new environment after shipment may aid to minimize the baseline activation of cells.

Upon confirming that the control strains do respond to LPS as expected, LPS-hyporesponsiveness of C3H/HeJ strains need to be verified, and compared to the level of LPS-induced maturation in by cells from the control strain(s). Readouts for LPS-hyporesponsiveness should include not only phenotypical assessments of DCs such as maturation marker expression but also functional assessments, including cytokine production by DCs in response to LPS and by T cells in response to LPS-treated DCs, as well as proliferation of allogeneic T cells in response to LPS-stimulated DCs from the LPS-hyporesponsive strain. Similar to the blocking of receptors using blocking antibodies, soluble forms of TLRs can be used to block TLR signaling; soluble forms of TLR4 have been reported in mice, and recombinant soluble form of this receptor decreased NFκB activation and TNCα release in response to *in vitro* LPS stimulation (Iwami et al., 2000). As multiple products of TLR4 mRNA exist in humans, soluble forms of TLR4 is thought to exist in humans as well, and may be used to block signaling mediated by membrane-bound TLR4 (Iwami et al., 2000). In addition, to reduce experimental variance, cells from individual animals may be pooled for replicates, rather than using cells from a given animal for a given replicate. Although pooling of cells is not appropriate for human studies, it is acceptable for this particular study as these animals are syngeneic. While these experiments focus on the elucidation of TLR4, other related candidates of receptors and molecules involved in biomaterial-induced DC maturation include MyD88 and TLR2. Another approach to studying the role of these receptors may be to transfect cells to stably express these receptors. Such studies using TLR2- or TLR4-transfected HEK293 cells have been describeded for other applications studying the role of these receptors (Kurt-Jones et al., 2002; Mandell et al., 2004).

As biomaterials introduced into protein-containing environment are instantaneously adsorbed with proteins, DC maturation in response to the presence of biomaterial is most likely mediated by such adsorbed protein layer. However, in the context of clinical applications, it is important to be congnisant of the absence of serum in DC preparations generated for clinical applications. In all of the *in vitro* studies presented in this thesis, DCs were cultured in presence of autologous plasma or FBS. Thus it may be worthwhile to determine *in vitro* whether biomaterial contact induces DC maturation in such serum-free environment. With this caveat, nevertheless, biomaterials integrated as a part of combination product are introduced into the host, where host proteins will be encountered. As such, identification of proteins adsorbed to the biomaterial may direct identification of receptors, other that mediate biomaterial-induced DC maturation or uptake of biomaterial MPs or degradation particles generated from a larger biomaterial implant. Given the wide spectrum of proteins that may adsorb to the biomaterial, it is likely that receptors other than or in addition to TLR family of receptors may be involved in the biomaterial-induced DC maturation. This approach of receptor identification may be more direct in that knowing the proteins adsorbed to the biomaterial, likely canddate receptors should emerge, as many of the protein ligand and receptor interactions as well as the ensuing effects on cellular behavior in the context of protein adsorption on biomaterials have been well characterized.

One significant factor in studying DC maturation is the presence of endotoxin. Content of endotoxin present in the biomaterial MPs and films used in the studies presented here were quantified using a commercially available chromogenic assay, and to the best of our ability, introduction of endotoxin was minimized through careful

procedures and use of materials designated as sterile and non-pyrogenic. While polymyxin B has been shown to inhibit LPS-induced DC activation, in our hands, titration of polymyxin B was unable to reduce LPS-induced increase in co-stimulatory marker expression (Figure A6, APPENDIX). This may have been due to purity of polymyxin B or LPS, inadequate reconstitution procedures, or insufficient range of concentrations tested relative to the amount of LPS present. Troubleshooting of this assay to be able to use polymyxin B to eliminate possibility of maturation due to presence of endotoxin would great simplify any additional studies investigating the biomaterial effect on DC maturation.

While this study evaluated the adjuvant effect of biomaterials focusing on the level of DC maturation, further development of assays to screen for biomaterials for potential applications in combination products may require a higher throughput approach. This would require the confirmation of correlation between the DC maturation readouts used in this study to other readouts that are translatable to high throughput systems such as mRNA and protein expression using genomics and proteomics, respectively. Techniques such as DNA microarrays (Ahn et al., 2002; Edwards et al., 2003) and serial analysis of gene expression (Hashimoto et al., 1999), as well as proteomics (Richards et al., 2002) have recently been applied to DCs. These techniques can assess a multitude of gene or protein expression simultaneously, making them indispensable for mass screening of identifying biomaterials that do or do not induce DC maturation, as well as for identification of signaling pathways involved in biomaterial-induced DC maturation. In addition, for the development of novel biomaterials and modifications to improve biomaterial compatibility, combinatorial libraries of chemical structures or high

throughput biomaterial systems with varying physicochemical properties associated with inducing certain cellular behavior may be developed, analogous to the identification of lead compounds used in drug development. This would provide a systemic approach to evaluating chemical properties of biomaterials such as hydrophilicity, hydrophobicity, charge, and presence of certain chemical end groups on cell behavior. For example, libraries containing multiple chemical modifications with increasing hydrophilicity may be screened for effects on DCs, and from such results, biomaterial modifications may be fine-tuned for desired outcome.

Research investigations presented in this thesis begin to address the differential immune and inflammatory responses associated with biomaterials in the context of combination products by focusing on the adjuvant effect at the level of DC maturation. Knowledge gained from this study combined with knowledge that may be gained from future studies may guide the development of biomaterial-centered strategies for the control of host immune response to address some of the current clinical limitations in regenerative medicine.

APPENDIX

A.1 Titration of phagocytosis inhibitors and their effects on DC maturation

To investigate the contribution of phagocytosis on DC maturation, pharmacological agents known to block phagocytosis or macropinocytosis were titrated. Cytochalasin D was used to inhibit phagocytosis as it disrupts actin filaments and inhibits actin polymerization (Flanagan and Lin, 1980; Cooper, 1987), and amiloride was used to inhibit macropinocytosis as it blocks Na^+/H^+ channels (Maidorn et al., 1993; Xiao and Allen, 1999). To find a working concentration for these agents to block MP internalization by DCs, varying concentrations of these agents were used. Human DCs on day 5 of culture were pretreated with indicated concentrations of cytochalasin D or amiloride for 1 hr at 37°C, and 3 μm FYG microspheres added at 1:1 MP:cell ratio for 4 hrs. Cells were then washed in FACS buffer without $CaCl_2$ and their fluorescence analyzed by flow cytometry. Both cytochalasin D and amiloride decreased DC uptake of fluorospheres in a dose-dependent manner (Figure A1a, A1b).

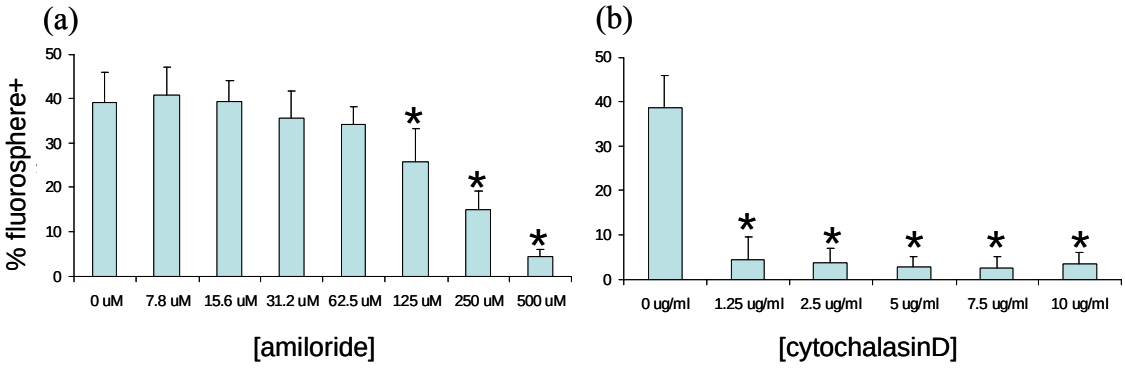

Figure A1: Dendritic cells treated with increasing concentrations of amiloride (a) or cytochalasinD (b) internalize decreasing numbers of fluorospheres.
mean±SD, n=4. *: $p<0.05$ compared to 0 μM amiloride or 0 μg/ ml cytochalasinD.

Contribution of phagocytosis on biomaterial-induced DC maturation was evaluated by pretreating DCs with the predetermined concentrations of cytochalasin D or amiloride, and then exposing DCs to 5:1 MPs: cell ratio of PLGA or agarose MPs, and assessing CD83 and CD86 expression. As shown in Figure A2, pretreatment of iDCs with cytochalasin D or amiloride without any additional maturation stimulants resulted in increased expression of CD83 and CD86. This result suggested that use of these inhibitors to assess DC maturation was not appropriate, as it would be difficult to determine the source of DC maturation if these agents alone induced increase in marker expression. To further evaluate the contribution of phagocytosis on DC maturation, biomaterial MPs of differing sizes were used, as described in CHAPTER 7.

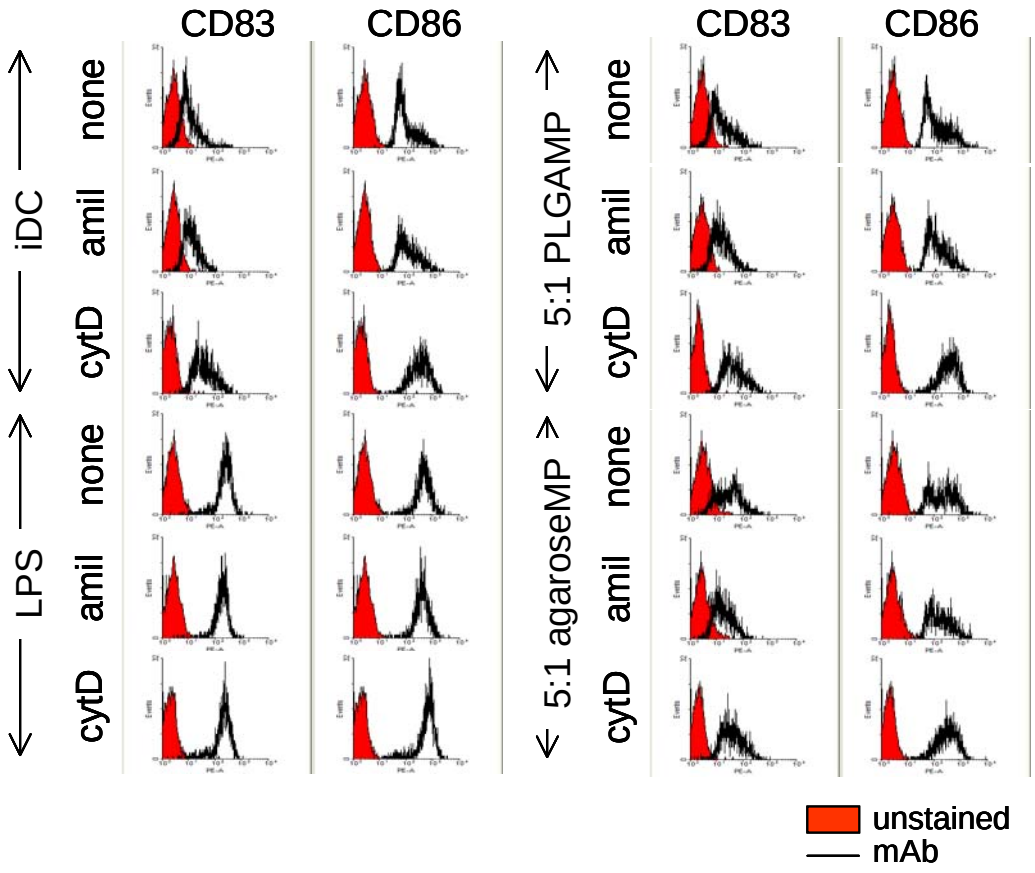

Figure A2: Phagocytosis inhibitors amiloride (amil) and cytochalasin D (cytD) alters DC marker expression.
This experiment was repeated three times with similar results, and representative results are shown.

A.2 Inflammatory cytokine profile of DCs treated with PLGA or agarose MPs

Profiles of inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNFα, and IL-12p70), released by DCs in response to treatment with PLGA or agarose MPs or film were evaluated using Cytometric Bead Array Human Inflammation kit (Becton Dickinson Pharmingen). Immature DCs were treated with the indicated materials (MPs at MP:cell ratios of 0.1:1, 1:1, 10:1, or with film) for 1 day, and the cell culture supernatants cleared and stored at -20°C until analysis according to the manufacturer's directions. In this assay, amounts of cytokine released are measured as fluorescence intensity, which is then converted into concentration units for each of the cytokines using a standard curve. As some of the cytokines were below or above the concentration range provided by the standard curve, data is shown as graphs of raw fluorescence values (Figure A3). In general, profiles of cytokines secreted by DCs treated by agarose or PLGA were similar. Pro-inflammatory cytokines, TNFα, IL-8 and IL-6, were released in a MP-dose dependent manner, while IL-12p70 was detected at a very low level, comparable to that released by iDCs. Dendritic cells treated with agarose or PLGA secreted low amounts of IL-10 and IL-1β. Although not a direct comparison, DCs exposed to PLGA generally secreted higher amounts of pro-inflammatory cytokines.

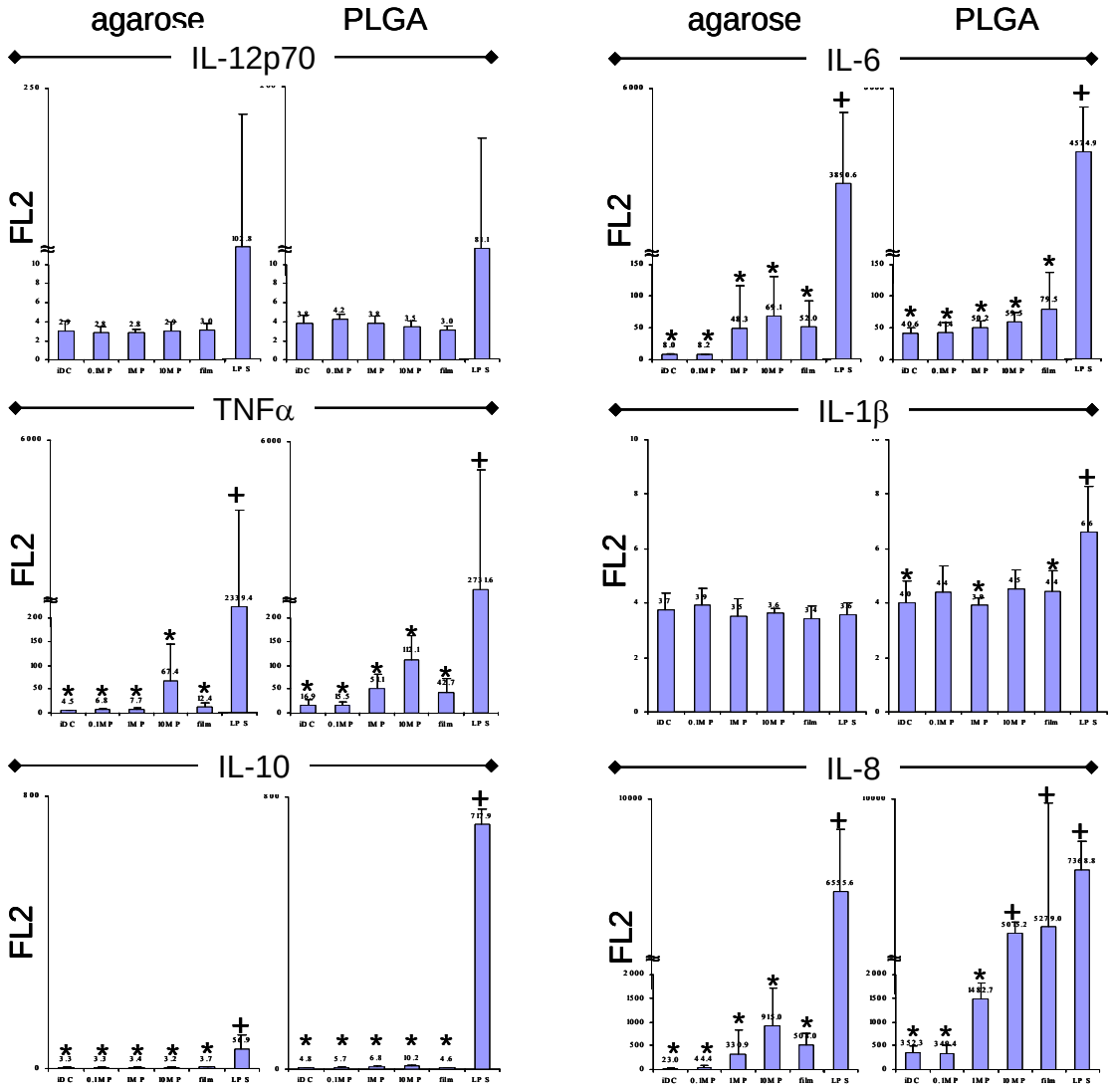

Figure A3: Dendritic cells treated with agarose or PLGA secrete inflammatory cytokines.

Data reported as raw fluorescent intensity values (FL2). MP:cell ratios indicated: 0.1MP= 0.1:1, 1MP= 1:1, 10MP= 10:1. mean±SD, n=3. *: $p<0.1$ compared to LPS, +: $p<0.1$ compared to iDC.

A.3 *In vitro* blocking of endocytic and signaling receptors on iDCs

In vitro blocking of endocytic and signaling receptors was performed to evaluate the involvement of particular receptors on PLGA-induced DC maturation. Receptors were chosen based on previous findings of their role in DC phagocytosis or maturation. Expression of CD11b (C3 complement receptor, Mac-1 subunit), CD32 (FcγRII), CD36 (scavenger receptor), CD51 (α_v integrin, combines with CD61 to form vitronectin receptor), CD61 (β_3 integrin, combines with CD51 to form vitronectin receptor, combines with CD41 to form gpIIb/gpIIIa complex), CD64 (FcγRI), DC-SIGN, Scavenger Receptor A, mannose receptor, and TLR2 and TLR4 was measured using flow cytometry (Figure A4).

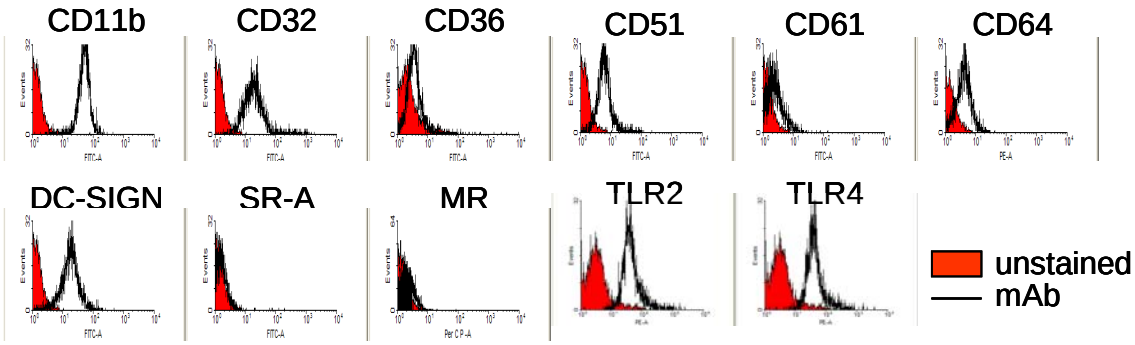

Figure A4: Immature DCs express endocytic and signaling receptors.
SR-A: scavenger receptor A, MR: mannose receptor, TLR2/4: Toll-like receptor 2/4. This experiment was repeated three times with similar results, and representative results are shown.

As reported in CHAPTER 8, *in vitro* blocking experiments were performed using blocking antibodies against CD36, CD51, DC-SIGN, and mannose receptor in addition to CD14, TLR2, and TLR4. For DC-SIGN and mannose receptors, both of which are lectin receptors, mannan was used as a ligand. For CD36 (scavenger receptor), polyguanylic acid (polyG) was used as a ligand, as CD36 binds polyanionic ligands; polycytidylic acid (polyC) was used as a negative control for polyG. As a ligand for CD51 (α_v integrin), arginine-glycine-aspartic acid (RGD) peptide was used. Blocking of receptors using antibodies or known ligands to these receptors within the range tested was not able to block the LPS- or PLGA MP-induced maturation, and in some situations increased the expression of CD83 (Figure A5a-c). Where ligands were used to block the receptors, endotoxin contents of ligands are indicated for each figure.

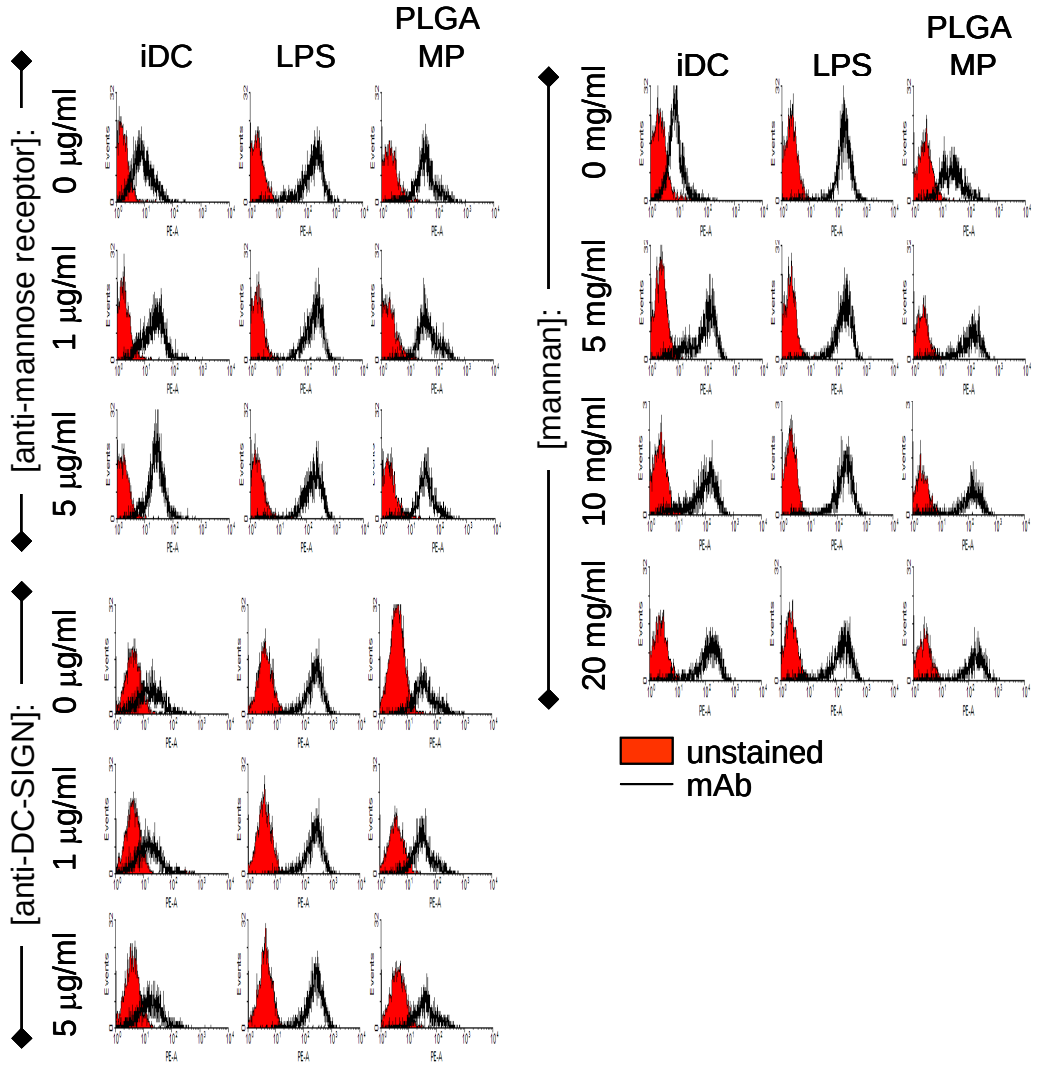

Figure A5a: PLGA MP-induced CD83 upregulation is not altered by increasing concentrations of anti-mannose receptor or anti-DC-SIGN antibodies, but is increased by increasing mannan concentration.
endotoxin contents: mannan: 3.056 EU/ ml (final)
This experiment was repeated three times with similar results, and representative results are shown.

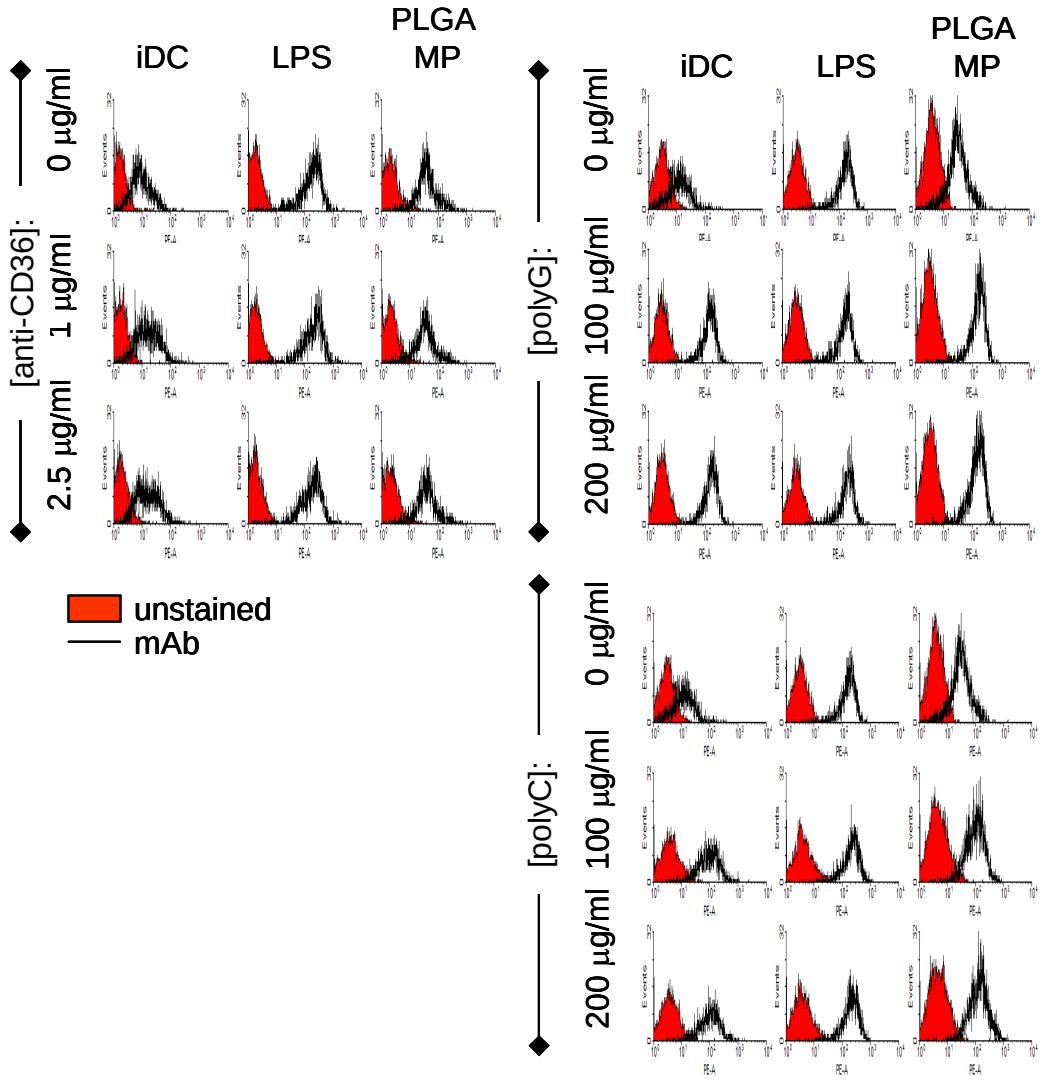

Figure A5b: PLGA MP-induced CD83 upregulation is not altered by increasing concentration of anti-CD36 antibody, but increased by increasing concentrations of polyG or polyC.
endotoxin contents: polyG: 0.0076 EU/ ml (final), polyC: 0.099 EU/ ml (final)
This experiment was repeated three times with similar results, and representative results are shown.

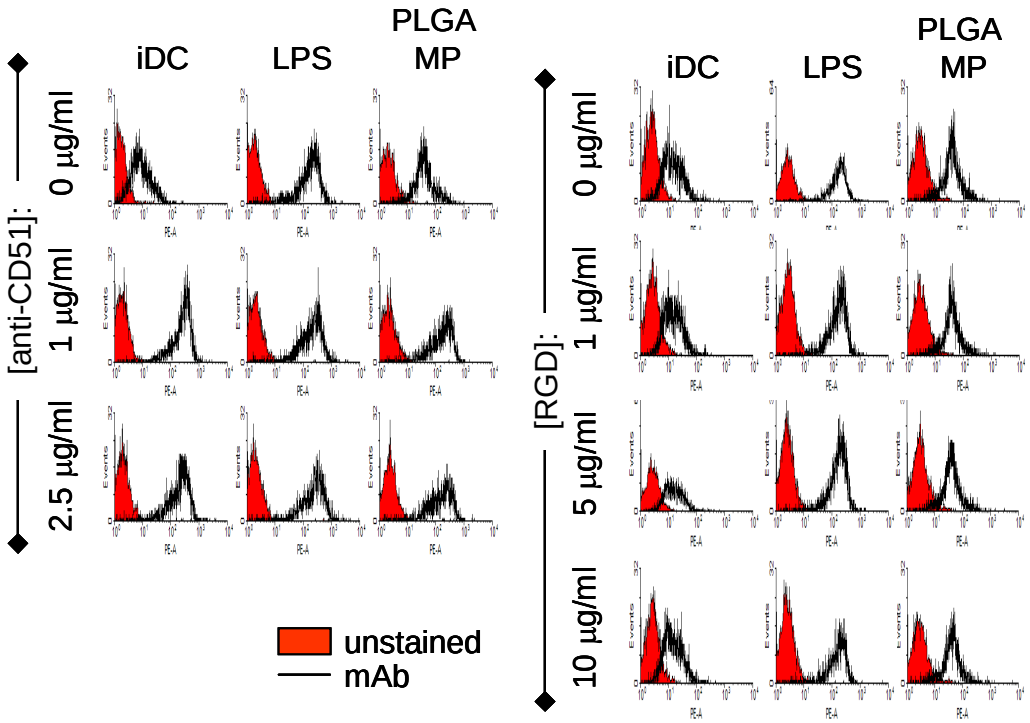

Figure A5c: PLGA MP-induced CD83 upregulation is increased by increasing concentration of anti-CD51 antibody, but not altered by increasing concentration of RGD (arginine-glycine-aspartic acid peptide).
endotoxin contents: RGD: <0.0001 EU/ ml (final)
This experiment was repeated three times with similar results, and representative results are shown.

A.4 Titration of polymyxin B to reduce LPS-induced DC maturation

To eliminate the possibility of DC maturation in response to presence of contaminating endotoxin in biomaterial preparations, addition of polymyxin B to DC culture was investigated. Polymyxin B is an antibiotic which binds to the anionic lipid A moiety of LPS, and neutralizes the effects of LPS (Morrison and Jacobs, 1976). Addition of polymyxin B alone has been shown to have no effect the expression of DC co-stimulatory and MHC molecules, while addition of polymyxin B to LPS-treated cells resulted in abrogation of upregulation of co-stimulatory and MHC molecules (Yang et al., 2002). In our hands, addition of polymyxin B to DC culture was not able to reduce LPS-induced increase in maturation marker expression (Figure A6). However, in our experiments, DC maturation was induced by PLGA, which contained endotoxin contents below the detection limit of the assay, whereas agarose, which had higher amounts of endotoxin, did not.

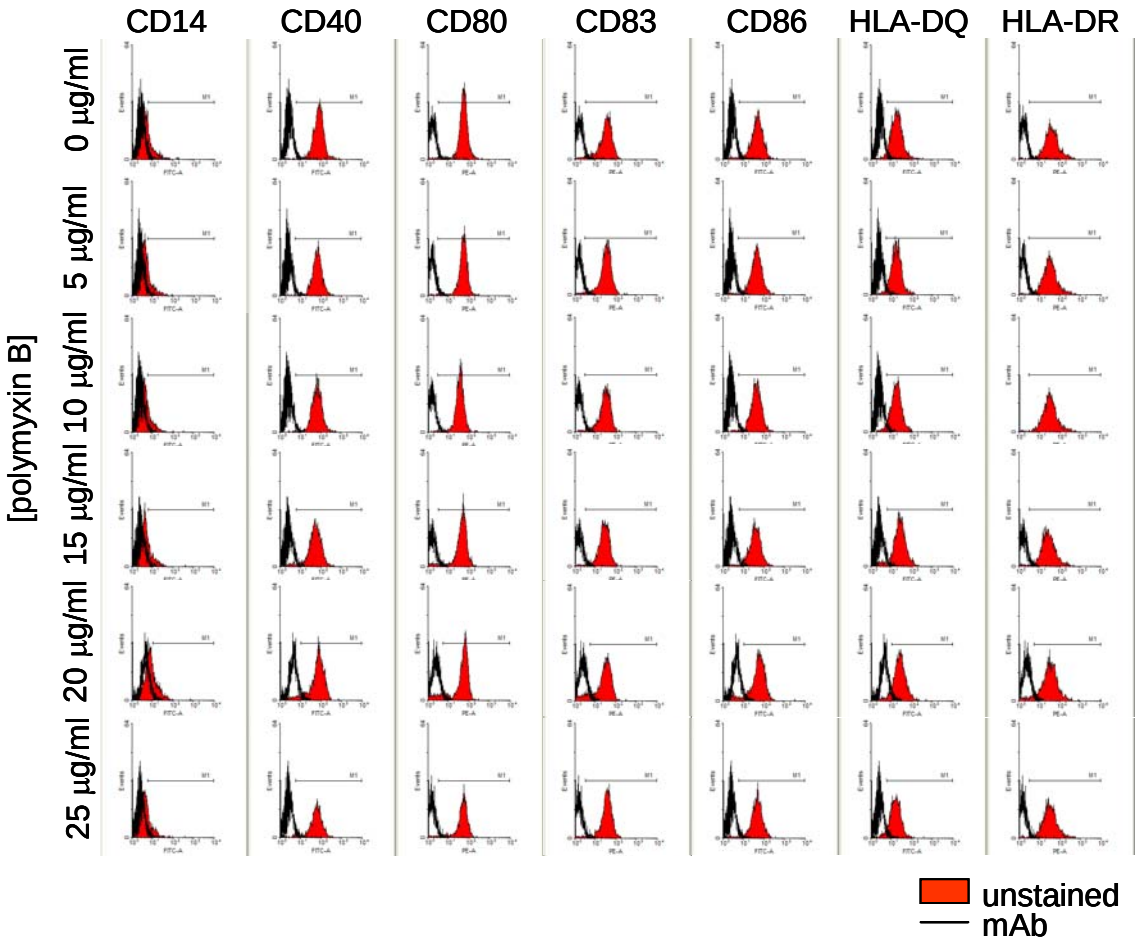

Figure A6: Dendritic cells treated with LPS in the presence of increasing polymyxin B concentrations do not alter their expression of maturation markers. This experiment was repeated three times with similar results, and representative results are shown.

A.5 Comparison of increase in DC maturation marker expression induced by Complete Freund's Adjuvant and LPS

To gauge the relative DC maturation effect of LPS, commonly used as a positive control for DC maturation *in vitro* to the adjuvant effect of Complete Freund's Adjuvant (CFA), commonly used adjuvant *in vivo*, DC maturation in response to CFA and LPS were compared *in vitro*. Human iDCs were left untreated or treated LPS, CFA emulsion (1:1 with PBS) (1 μl/ ml or 10 μl/ ml), or with PLGA MPs, and expression of co-stimulatory and MHC molecules measured to gauge the extent of maturation induced by CFA. No DC maturation as measured by expression of co-stimulatory and MHC molecules was observed for these CFA-treated DCs (Figure A7). This lack of increase in maturation markers may be due to the difficulty in delivering CFA emulsion *in vitro* because of its hydrophobicity (formation of floating clumps on surface of cell culture well). A modification was made to this experiment, and CFA was used without any emulsification (without any dilution). At concentrations tested (1 μl CFA/ ml cell culture, 10 μl CFA / ml cell culture, 100 μl CFA/ ml cell culture), dose-dependent increase in the expression of co-stimulatory and MHC molecules were observed (Figure A8). However, even at the highest dose of 100 μl CFA/ ml cell culture, the extent of maturation induced as measured by the expression levels of these surface markers was consistently lower than that induced by 1 μg/ ml LPS, positive control used for all of our other studies.

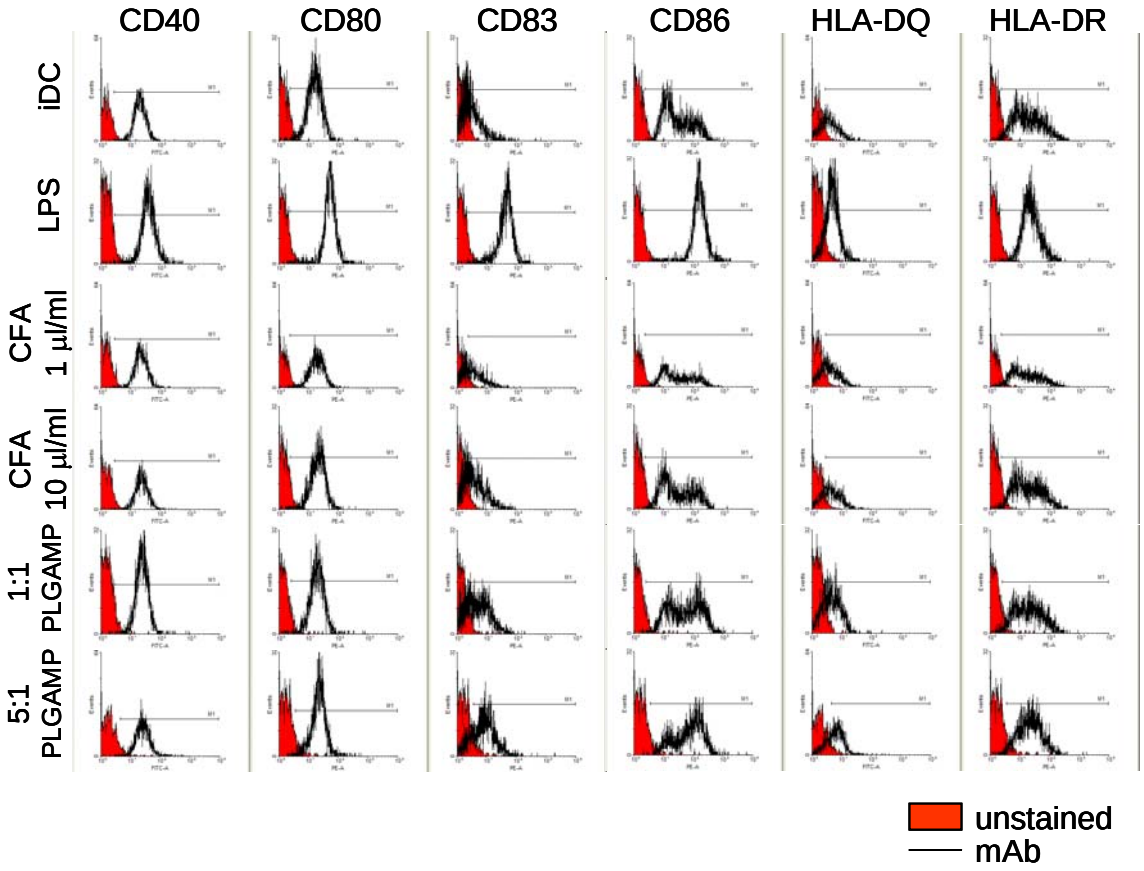

Figure A7: Increase in DC maturation marker expression in response to CFA is lower than that induced by PLGA MPs.
This experiment was repeated three times with similar results, and representative results are shown.

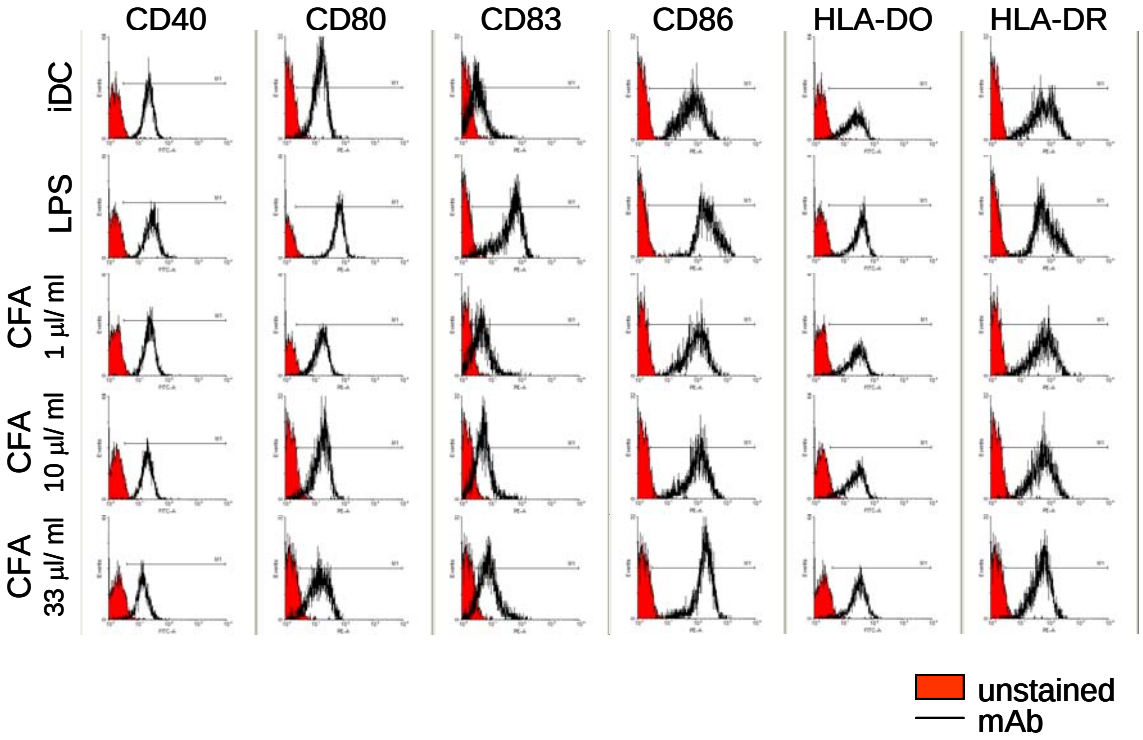

Figure A8: Increase in DC maturation marker expression in response to CFA is lower than that induced by LPS.
This experiment was repeated three times with similar results, and representative results are shown.

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